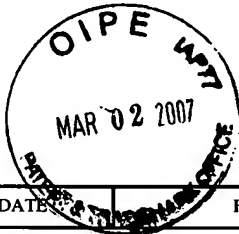




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Familial and sporadic inflammatory bowel disease: comparison of clinical features and serological markers in a genetically homogeneous population.

Halme L, Turunen U, Helio T, Paavola P, Walle T, Miettinen A, Jarvinen H, Kontula K, Farkkila M.

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BACKGROUND: The familial occurrence of inflammatory bowel disease (IBD) and the clinical features of familial and sporadic IBD in the genetically homogeneous Finnish population are evaluated. **METHODS:** 257 patients with Crohn disease (CD) and 436 with ulcerative colitis (UC) participated in the study. They were asked whether IBD was present (familial IBD) or absent (sporadic IBD) in their first-degree relatives. Data on the clinical course of the disease were collected from the patient records. Antibodies to *Saccharomyces cerevisiae* (ASCA) and anti-neutrophil cytoplasmic antibodies (ANCA) were determined from serum samples. **RESULTS:** Affected first-degree relatives were found in 15.6% of patients with CD and in 13.8% of patients with UC. In familial cases CD was more often located in the ileum (38% versus 21%) and less often in the ileocolon (35% versus 50%) ($P < 0.05$) than in sporadic cases. A greater percentage of CD patients than UC patients were smokers (47% versus 13%; $P < 0.01$). An elevated level of IgA and/or IgG antibodies for ASCA was found more often in CD patients than in UC patients (59% versus 14%; $P < 0.01$), while pANCA were found more often in UC than in CD patients (48% versus 12%; $P < 0.01$). The combination of pANCA-ASCA+ yielded a sensitivity, specificity and positive predictive value of 48%, 92% and 90%, respectively, for CD, and the combination of pANCA + ASCA- of 55%, 94% and 90%, respectively, for UC. **CONCLUSIONS:** The percentage of familial IBD cases in Finland is comparable to that reported elsewhere in Europe. No important clinical differences between patients with familial and sporadic forms of the disease were found. ASCA is associated with both familial and sporadic CD and pANCA with UC, but low sensitivity diminishes their value as a serological marker of IBD or as a differential diagnostic test between CD and UC.

☐ 1: MLO Med Lab Obs. 2001 Nov;33(11):8-15; quiz 16-9.

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Serologic markers in inflammatory bowel disease (IBD).

Nakamura RM, Barry M.

MLO Medical Laboratory Observer

Prometheus Laboratories, San Diego, CA, USA.

Inflammatory bowel disease (IBD) is a generic term that refers to Crohn's disease and ulcerative colitis. Crohn's disease (CD) is a granulomatous enteritis which can involve the ileum, colon, and other parts of the intestinal tract. The serologic responses seen in Crohn's disease include antibodies to *Saccharomyces cerevisiae*, mycobacteria, bacteroides, listeria and *E. coli*. Many of these organisms may be involved in the pathogenesis of the Crohn's disease. Ulcerative colitis is characterized by inflammation of the mucosa and submucosa of the large intestine. The CD and UC are considered to be distinct forms of IBD; however, there is a subgroup of CD with a UC-like presentation. In recent years, several serologic markers have been found to be useful for the diagnosis and differentiation of CD and UC. These markers include the following antibodies (a) 2pANCA, (b) ASCA, (c) pancreatic antibody, and (d) OmpC antibody. The application of a panel of markers with the use of an algorithm can identify specific subtypes of IBD that have different clinical courses and progression of the diseases. The application of the serologic markers is

Serologic Markers in Inflammatory Bowel Disease (IBD)

By Robert M. Nakamura, MD and
Mary Barry, MBA, MT(ASCP)

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LEARNING OBJECTIVES

Upon completion of this article
the reader will be able to:

1. Differentiate between inflammatory bowel disease and irritable bowel syndrome.
2. Name at least three serum immune markers that are useful in the diagnosis and management of irritable bowel syndrome.
3. Describe the origin(s) of at least three serum immune markers identified in question 2.
4. Explain the types and limitations of methods used in detection and quantification of serological markers of IBD.

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Inflammatory bowel disease (IBD) is a generic term that refers to Crohn's disease and ulcerative colitis. IBD is prevalent in children as well as adults. Crohn's disease (CD) is a granulomatous enteritis which may involve the ileum, colon, and other parts of the intestinal tract. Crohn's disease was first reported by Burrill Crohn and his colleagues in 1922 and was called regional ileitis.¹ Crohn's disease is diagnosed in at least four patients per 100,000 in the United States, and the incidence and prevalence is rising.²

Ulcerative colitis (UC) is a chronic disease of unknown etiology characterized by inflammation of the mucosa and submucosa of the large intestine. The inflammation usually involves the rectum down to the anal margin and extends proximally in the colon for a variable distance. Ulcerative colitis may have a prevalence of about 100 cases per 100,000 population in the United States.³

Overlap of Crohn's disease and ulcerative colitis

Crohn's disease (CD) and chronic ulcerative colitis (UC) are generally considered to be distinct forms of inflammatory bowel disease (IBD). However, the symptoms and clinical presentations of CD and UC commonly overlap, and the diagnostic differentiation of cases limited to the large intestine may be problematic.^{4,5} There is a subgroup of cases of CD with a UC-like presentation that illustrates the similarity of CD and UC.^{5,7} A patient who may have been initially diagnosed as having UC may over time be considered as a case of CD in view of extension of the disease.^{8,9}

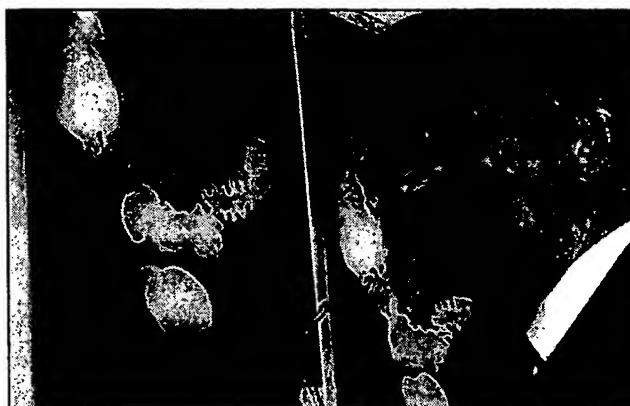
Today, new and improved therapeutic modalities are available for CD and UC. As these various cases of IBD are treated with different types of therapeutic agents, it is important to correctly diagnose IBD and to differentiate CD from UC.

What is classically termed Crohn's disease (CD) may represent a heterogeneous group of diseases manifesting similar features.¹⁰⁻¹³ A recent study suggests that Crohn's patients may have a heterogeneous serological response to specific bacteria and bacterial related antigens.¹⁴ The serologic responses seen in Crohn's patients include antibodies to *Saccharomyces cerevisiae*, *mycobacteria*, *bacterioides*, *listeria*, and *E. coli*.

Many of the specific organisms have been proposed to directly or indirectly contribute to the pathogenesis of Crohn's disease.¹⁴

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is the most common functional disorder of the gastrointestinal tract. The hallmark of IBS is abdominal pain or discomfort associated with a change in the consistency or frequency of stools.^{15,16} IBS is differentiated from IBD, as IBD is associated with an inflammatory response. IBS occurs at a frequency of 8 percent to 23 percent in adults in the Western world.^{10,16} For IBS, there is at present no specific diagnostic laboratory test and there is an absence of definite biological markers. The diagnosis of IBS is based on a constellation of symptoms. An international consensus group has developed criteria for IBS in an effort to standardize the definition of IBD.^{12,18} The criteria have been termed the "Rome Criteria" in reference to the location of the meeting. The criteria have been further refined.¹⁵



Immune markers associated with IBD

During the past few years, several investigators have identified diagnostic serological markers for UC and CD. Drs. S. Targan and J. Braun and their respective groups of investigators have made a significant contribution. The serologic markers discussed below have been found to be useful for the diagnosis and differentiation of CD and UC. In addition, a panel of markers with use of an algorithm can identify specific subtypes of IBD that have different progressions and clinical courses. Thus, the panel of serologic markers are useful for diagnosis and management of CD and UC patients.

Classification of serum immune markers in IBD

Currently the following serum immune markers have found to be useful for diagnosis and management of IBD:¹⁹⁻²³ (see Chart 1)

- 1) Deoxyribonuclease (DNase I) sensitive perinuclear antineutrophil cytoplasmic autoantibody (pANCA) associated with IBD. The IBD associated pANCA defines an antibody to a nuclear antigen which is sensitive to DNase I.
- 2) ASCA (Anti-*Saccharomyces cerevisiae* antibody).^{21,26} This antibody is present in the serum of up to 70 percent of Crohn's disease patients.
- 3) Pancreatic antibody.²⁷⁻²⁹ This antibody is observed in approximately 30 percent of Crohn's disease patients. Two distinct staining patterns have been reported.
- 4) Anti-OmpC (outer membrane porin from *E. coli*).³⁰⁻³¹ An IgA response to OmpC is seen in 55 percent of Crohn's disease patients.

Significance of ANCAs

(Anti neutrophilic cytoplasmic antibody)

• **ANCAs associated with vascular diseases (vasculitides).** ANCAs are autoantibodies directed against the intracellular components of the neutrophils. Over the past decade, ANCA has received considerable attention as it was seen in inflammatory vasculitides.³²⁻³⁵ The key antigen in the cytoplasm of neutrophils was found to be serine proteinase 3.³⁶ The staining of ANCA reaction with proteinase 3 will result in a cytoplasmic fluorescent pattern called c-ANCA. The pANCA pattern of perinuclear staining around the nuclei of neutro-

Continues on page 10

Chart 1

CD COHORT							ULCERATIVE COLITIS COHORT						
	Total	ASCA Panel+	IgA OmpC+	pANCA+	% Detected	Cumulative % Detected		Total	ASCA Panel+	IgA OmpC+	pANCA+	% Detected	Cumulative % Detected
Entire Cohort	175	96			54.9%	54.9%	Entire Cohort	100			68	68.0%	68.0%
ASCA Panel -	79		22		12.6%	67.4%	pANCA neg	32		2		2.0%	70.0%
ASCA Panel -/OmpC-	57			15	8.6%	76.0%	pANCA -/OmpC-	30	2			2%	72.0%
ASCA Panel -/OmpC- /pANCA-	42				0.0%	76.0%	pANCA -/OmpC- /ASCA Panel -	28				0.0%	72.0%
Total % detected:					76.1%		Total % detected:					72.0%	
IBD COHORT							NORMAL, IBS, DISEASE CONTROLS						
Entire Cohort	275	104			37.8%	37.8%	Entire Cohort -	127	1			0.8%	0.8%
ASCA Panel -	171		32		11.6%	49.5%	ASCA Panel -	126		3		2.4%	3.1%
ASCA Panel -/OmpC-	139			69	25.1%	74.5%	ASCA Panel -/OmpC-	123			4	3.1%	6.3%
ASCA Panel -/OmpC- /pANCA-	70				0.0%	74.5%	ASCA Panel -/OmpC- /pANCA-	119				0.0%	6.3%
Total % detected:					74.5%		Total % detected:					6.3%	

phils is the second type of pattern noted. The pANCA pattern is the result of positively charged molecules that migrate to the edge of the nuclei of neutrophils. This phenomenon occurs after alcohol fixation of the substrate cells. The cytoplasmic granules redistribute around the nuclei, resulting in a pANCA pattern in case of antibodies to elastase, lactoferrin, cathepsin G, and myeloperoxidase. The pANCA pattern with myeloperoxidase is significant since antibodies to myeloperoxidase may be seen in vasculitis.³⁶ Antibodies to serine proteinase 3 and myeloperoxidase may be specifically detected by an ELISA (enzyme linked immunosorbent assay).

• **Association of ANCA with inflammatory bowel disease.** IBD associated ANCA was first reported in 1966 by Faber and Elling, who described "leukocyte-specific antinuclear antibodies" in patients with Crohn's disease and ulcerative colitis.³⁸ It is now clear that the granulocyte specific antinuclear antibodies are in fact pANCA.³⁹ Many investigators have subsequently noted the association of pANCA with IBD.^{5,10,25,39} The reported incidence of serum ANCA in UC patients was reported to be between 50 and 80 percent.¹⁹⁻²³ Serum pANCA is believed to reflect mucosal pANCA production in some instances. Studies have shown that pANCA production takes place in the colonic mucosa.^{19,20} It appears that the mucosal antigens lead to local production of pANCA in the intestinal tract.

• **pANCA associated with ulcerative colitis.** The majority of adult patients with UC (60 percent to 80 percent) exhibit a positive test for pANCA.¹⁹ Also pANCA has been observed in 83 percent of children.¹⁹ Billing et al. have provided evidence that the pANCA antigen associated with UC is nuclear in location.⁴⁰ They studied the neutrophil reaction with confocal and electron microscopy and demonstrated that the UC-associated pANCA reaction was localized primarily over chromatin concentrated toward the periphery of the nuclei.⁴⁰ The UC patients' sera also did not recognize double stranded DNA. There may be multiple antigens and epitopes involved in the atypical pANCA and it has been reported as associated with histone-1,^{41,42} high mobility group nuclear protein (HMG-1 and HMG-2),^{43,44} and more recently as a 50 kilodalton nuclear envelope protein.⁴⁵

The pANCA pattern seen in IBD is the result of nuclear antigens which are DNase I sensitive. The pANCA stain-

ing pattern is lost after the DNase I digestion of the substrate cells. In approximately 70 percent of the cases of UC, there is ablation of the pattern and antigen recognition, and in up to 30 percent of the cases there is conversion to homogeneous cytoplasmic staining.¹⁹⁻²³ In 3 percent of UC patients evaluated displaying pANCA reactivity, the pANCA pattern was retained after DNase treatment of the substrate.¹⁹⁻²³ The retained pattern may represent concurrent antibodies present to cytoplasmic or nuclear antigens other than the UC associated pANCA antigen. ANCA are present in the sera of 60 percent to 80 percent of patients with ulcerative colitis and 10 percent to 30 percent of patients with Crohn's disease. Eighty-three percent of children and adolescents with ulcerative colitis showed the expression of ANCA in their sera.²³

Various studies have shown that UC patients with pANCA represent subpopulations which show production of pANCA. This may be consequence of a distinct mucosal inflammatory process.

What does the expression of pANCA mean in patients with UC?

The pANCA expression allows for stratification of the UC patients at the clinical and genetic levels. In adults, clinically distinct subsets of UC have been observed based on the presence of ANCA/pANCA as these patients have a higher probability of:

- Having left-sided ulcerative colitis which is more resistant to treatment than the usual case.
- Having more aggressive disease.
- Requiring surgery early in the course of the disease.
- Developing pouchitis in UC following ileal pouch-anal anastomosis.
- Having specific HLA markers.

The serum pANCA in UC patients may reflect mucosal pANCA production. This suggests that recognition of mucosal antigens leads to local production of pANCA.

Targan and Braun and co-workers have recently demonstrated the presence of specific bacterial and/or bacteria-like antigens in patients with Crohn's disease and ulcerative colitis that appear to elicit antibody response in those patients.^{46,47}

With the use of phage display technology, monoclonal antibodies that cross-react with antigens that are similar to those seen in sera of UC patients who are pANCA

Continued on page 111

positive. These monoclonal antibodies cross react with bacterial antigens from *E. coli* and *bacterioides*.³⁰

Segregation of pANCA by DNase I treatment to differentiate ulcerative colitis from Type I autoimmune hepatitis and primary sclerosing cholangitis

UC associated ANCA yields a perinuclear staining pattern pANCA with methanol fixed neutrophils. pANCAs have been detected in the serum of patients with autoimmune hepatitis (Type I AIH), primary sclerosing cholangitis (PSC), and other autoimmune liver diseases.

The pANCA pattern has been identified in about 70 percent of ulcerative colitis (UC) patients. Also, the pANCA pattern with alcohol fixed neutrophils has been reported in 92 percent of sera from patients with well-defined Type I autoimmune hepatitis. Furthermore, the pANCA pattern was noted in up to 70 percent of PSC patients.

The pANCA associated with UC reactive antigen was associated with epitopes within the nuclei. In addition, the UC pANCA demonstrated loss of antigenic recognition after DNase I enzyme digestion of neutrophils as a dominant feature.

In direct contrast, the majority of Type I autoimmune hepatitis and PSC patients showed a pANCA pattern recognizing cytoplasmic constituents. Thus, the UC associated pANCA with epitopes within the nuclei is highly specific for inflammatory bowel disease.^{35,46}

Association of serum pANCA with subgroup of Crohn's disease

The serum pANCA are seen in 10 percent to 30 percent of patients who have diagnosed as having CD.¹⁹ In CD, expression of pANCA identifies a subgroup of CD characterized as "ulcerative colitis-like" phenotype in which patients have clinical features of left-sided colitis with histopathologic features of UC. The serum immunoglobulin IgG (immunoglobulin G) of pANCA positive CD patients is similar to the pANCA seen with UC patients. The presence of pANCA in both CD and UC suggests that there is a specific type of mucosal inflammation that may be common to CD and UC.^{19,22,48}

The CD patients who are pANCA positive did not respond as well as the majority of CD patients to anti-TNF (tumor necrosis factor) monoclonal antibody therapy. On the other hand, 65 percent of Crohn's disease patients responded well to anti-TNF monoclonal antibody therapy.¹⁹

High levels of pANCA in Crohn's disease patients were associated with later age of onset and an UC-like inflammatory response, as well as a relative decreased incidence of fibrostenosis and penetrating disease.²²

Method of assay for serologic markers in IBD

• Indirect Immunofluorescence Assay for ANCA.²⁵

Neutrophils were isolated from peripheral blood of normal persons by Ficoll-Hypaque density centrifugation followed by dextran sedimentation.⁴⁹

The neutrophils were resuspended in phosphate buff-

ered saline (PBS), and 100,000 cells were prepared on slides by cyto centrifugation. The slides were fixed in 100 percent methanol at 4°C for 10 minutes, air dried, and stored at -20°C. After incubation of the sera on the slides for 20 minutes, the slides were washed in PBS and stained with fluorescein-labeled F(ab'), gamma-chain specific antibody. After washing, the slides were examined by fluorescence microscopy.

• Formalin fixation and pitfalls in the assay for ANCA.⁵⁰

The British Association of Clinical Pathologists suggested that formalin acetone fixation followed by absolute ethanol may be useful to differentiate pANCA from ANA.⁵¹ This procedure has not been confirmed by international consensus. Lee et al.⁵² suggested that formaldehyde vapor fixation may be used to detect conversion of pANCA (pattern noted by alcohol fixation) to the cytoplasmic pattern when myeloperoxidase antibodies were present. However, Lee et al. found that formalin acetone fixation gave inconsistent results.⁵² Other studies have shown that formalin fixation caused inconsistency, nonspecific effects and false positivity owing to enhanced fluorescence.⁵³

In some cases neutrophils were incubated with the pANCA positive sera and the F(ab'), gamma-chain specific antibody before DNase I digestion of the slides.

The pANCA which are DNase I sensitive are characteristically seen in UC patients and CD patients. When the pANCA pattern is observed in UC patients and treated with DNase I, in 70 percent there is ablation of antigen recognition, and in 30 percent there is conversion to a homogeneous pattern.¹⁹

Continues on page 12

Chart 2

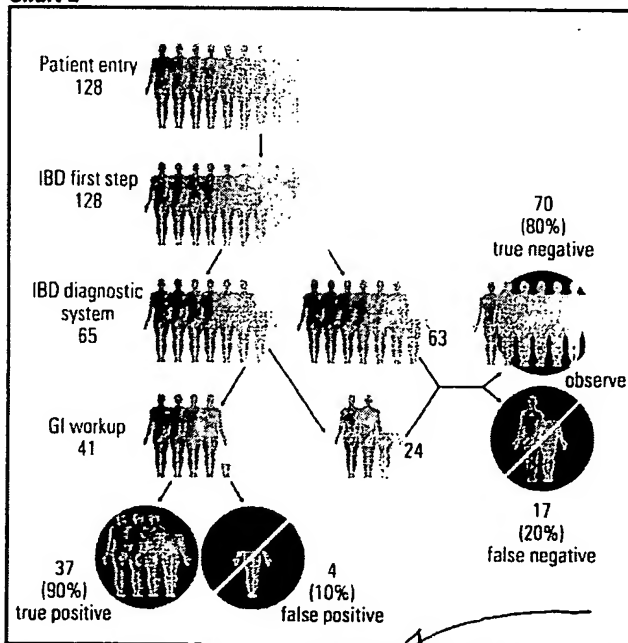


Chart shows predictive accuracy of the ASCA and pANCA IBD First Step and Confirmatory system performed on 128 pediatric patients by Promethias. Use of this test system would have avoided unnecessary invasive workup in 70 true negatives. Only 10 percent of patients would have been subjected to unnecessary workup. Overall diagnostic accuracy 107/128 (84 percent) Dubinsky, M. Serodiagnostic Testing in suspected pediatric patients.

- **Fixed neutrophil ELISA assay for ANCA.** The assay has been described by Saxon et al.²⁵ The microtiter plates were coated with 2.5×10^5 neutrophils per well. These were fixed in methanol for 10 minutes and allowed to air dry. To minimize nonspecific binding, 0.25 percent bovine serum albumin was added as a blocking agent.

The control patients' sera were added to different wells at dilution of 1:100. The neutrophil bound antibody was detected by alkaline phosphatase conjugated goat anti-human IgG. After addition of p-nitrophenol, specific absorbance was read at 405 nm. The levels of ANCA are determined relative to a laboratory standard expressed as ELISA Units (EU/mL). The standard was obtained from sera of pANCA positive patients with well-characterized UC.

- **ELISA assay for ASCA in CD patients.**^{22,48} The microtiter plates were coated with phosphopeptidomannans from the yeast *Saccharomyces cerevisiae* ssp *uvarum*. The control and patients' serum samples were added to different wells at a 1:100 dilution. Bound antibodies were detected with goat anti-human IgG and IgA labeled with alkaline phosphatase. After adding p-nitrophenol, the specific absorbance was measured at 405 nm. The absorbance of each serum sample was evaluated and assigned ELISA Units (EU/mL) values relative to the absorbance of a pool of sera collected from well-characterized patients with CD. The standard pool was arbitrarily assigned the value of 100 EU/mL. The result of the CD diagnostic system panel determines relative positivity of IgA and IgG ASCA respectively.

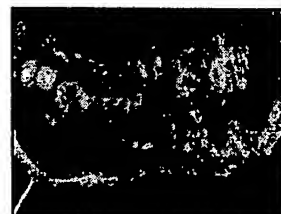
- **IBD first step screen for ANCA, IgG ASCA IgA ASCA and IgA anti OmpC.** Prometheus Laboratories developed quantitative tests that detect serum markers consistent with the presence of IBD.⁵⁴ This IBD First Step system consists of a set of four quantitative ELISA assays used together to detect ANCA, IgG ASCA IgA ASCA and IgA anti OmpC antibodies. The panel of assays shared a test sensitivity of 94 percent. The negative predictive value is greater than 95 percent when the data is modeled for an IBD prevalence commonly seen in a standard gastroenterology practice of 15 percent. The tests have a high sensitivity, but a much lower specificity. The main purpose of the test panel is to help rule out the presence of IBD. The algorithm with use of the sensitive modified assay is shown in Chart 2. The algorithm can be applied to children, adolescents and adults with IBD.

Variability of assays for ANCA and ASCA in different clinical laboratories. Sandborn, W. J. et al.⁵⁵ conducted a study with the purpose of evaluating serological markers in a population-based cohort of patients with ulcerative colitis and Crohn's disease. Blood and sera were obtained from 162 patients who agreed to participate in the study from a group of 290 IBD patients. Of the 162 patients, 83 had ulcerative colitis and 79 had Crohn's disease. The conclusions reached by the Sandborn et al. study:

- 1) The sensitivity of the ANCA assays varied widely in different laboratories.
- 2) The prevalence of ASCA was similar in the various laboratories participating in the study.
- 3) The positive predictive values of the ANCA and ASCA for the diagnosis and evaluation of UC or CD are high enough to be clinically useful.

Antibodies associated with Crohn's disease

Besides the pANCA that identifies a subgroup population of Crohn's disease, there are several other antibodies that are associated with Crohn's disease. These antibodies include *Saccharomyces cerevisiae* antibody (ASCA), pancreatic antibody, and antibody to OmpC (outer membrane porins isolated from *E. coli* bacteria).



Crohn's disease of small intestine shows hyperemia and focal areas of ulceration.

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ASCA

ASCA is a serum immune marker, which has been shown to be expressed in the majority of sera of CD patients.^{22,23,48} The ASCA antibodies have a high specificity for Crohn's disease.^{22,48} Serum ASCA is expressed in up to 70 percent of CD patients.

Small bowel disease was present in almost all CD patients who were positive for both IgA and IgG ASCA but negative for pANCA. The majority of patients in the subgroup may have signs of small bowel obstruction and perforating disease. The CD patients with IgA ASCA and IgG ASCA appear to have a more aggressive type of CD.

The ASCA assay is performed by an ELISA method. It should be emphasized that negative tests of pANCA or serum ASCA do not rule out the presence and diagnosis of IBD. The positive tests provide evidence that the patients with IBD should be evaluated further.^{48,52}

An ASCA ELISA with lower threshold was able to detect 90 percent of diagnosed Crohn's disease patients. When evaluated at the lower threshold to allow exclusion of IBD as a probable diagnosis for negative samples, a positive result must be followed up with more specific test to allow probable diagnosis of IBD.⁵²

Pancreatic antibodies in Crohn's disease

Pancreatic antibodies as detected by an indirect immunofluorescence test with human pancreas substrate occurred in 31 percent to 39 percent in Crohn's disease patients.²⁷ Of 212 CD patients studies, 30 patients had pancreatic antibodies characterized by a "drop-like" fluorescence in the pancreatic acini (subtype I).^{28,29} Twenty-eight patients demonstrated a fine speckled staining in the acinar cells of the pancreas (subtype II).^{28,29} Siebold et al.²⁸ concluded that pancreatic antibodies are specific markers for CD. Two subgroups were seen with different immunofluorescent patterns.

It remains to be determined whether the presence of the pancreatic antibody is associated with a defined sub-

Continues on page 14

group of CD patients. The specific antigen reacting with the pancreatic antibody has not been identified. These antibodies were rarely seen to occur in family members of patients with Crohn's disease.

The relevance of pancreatic antibodies in the pathogenesis of Crohn's disease is unclear. Stocker, et al. have reported that in patients with CD diagnosed for less than 2.5 years, the prevalence of pancreatic antibodies was 25 percent. However, if the CD existed longer than 2.5 years, the incidence of pancreatic antibodies was 46 percent.

Whether the presence of pancreatic antibodies in CD identifies a subgroup of Crohn's patients remains to be determined.

The previous reports used a substrate of human type O negative pancreatic tissue. One may be able to employ primate pancreatic tissue substrate. Siebold, et al. have observed that pancreatic tissue from rats and mice showed immunofluorescent patterns similar to that observed in human. Extensive comparative data of humans and rat tissue runs was not presented.

OmpC antibody in Crohn's disease

OmpC is an outer membrane porin antigen purified from *E. coli*.³⁰ ELISA assay with human sera demonstrated elevated IgG anti-OmpC in ulcerative colitis patients com-

pared to healthy controls.

Cohavy et al.³⁰ performed experiments on the hypothesis that pANCA identifies a bacterial antigen found in the human colonic mucosa. In these experiments a monoclonal pANCA antibody was used.³⁰ The ANCA monoclonal antibody was cloned by a phage display method and characterized.

The pANCA monoclonal antibody was reactive with *bacterioides* and *E. coli* antigens. The *E. coli* protein was biochemically and genetically identified as the outer membrane porin OmpC.

In patients with Crohn's disease, IgA response to OmpC was found in 55 percent of 151 patients, 56 percent were seropositive to ASCA, and 24 percent were positive with the pANCA test.

The serological response to the OmpC and panel of antigens studies by Landers et al.³¹ identified more Crohn's patients. There may be patient subsets of Crohn's that demonstrate variable responses to selected bacterial antigens.

Summary

Inflammatory bowel disease (IBD) is a generic term that refers to Crohn's disease and ulcerative colitis. Crohn's disease (CD) is a granulomatous enteritis which can in-

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volve the ileum, colon, and other parts of the intestinal tract. The serologic responses seen in Crohn's disease include antibodies to *Saccharomyces cerevisiae*, *mycobacteria*, *bacteroides*, *listeria* and *E. coli*. Many of these organisms may be involved in the pathogenesis of the Crohn's disease.

Ulcerative colitis is characterized by inflammation of the mucosa and submucosa of the large intestine.

The CD and UC are considered to be distinct forms of IBD; however, there is a subgroup of CD with a UC-like presentation.³⁴

In recent years, several serologic markers have been found to be useful for the diagnosis and differentiation of CD and UC. These markers include the following antibodies (a) pANCA, (b) ASCA, (c) pancreatic antibody, and (d) OmpC antibody. The application of a panel of markers with the use of an algorithm can identify specific subtypes of IBD that have different clinical courses and progression of the diseases. The application of the serologic markers is useful for diagnosis and management of CD and UC patients. □

Dr. Robert M. Nakamura serves as Laboratory Medical Director at Prometheus Laboratories in San Diego, CA. Mary Barry is Vice-president of Operations at Prometheus Laboratories, a small volume laboratory serving gastrointestinal specialists throughout the U.S.

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CE test on serologic markers in inflammatory bowel disease

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This test was prepared by Sharon M. Miller, PhC, CLS(NCA), MT(ASCP), Associate Dean and Shirley Richmond, EdD, CLS(NCA), MT(ASCP), Dean, College of Health and Human Sciences, Northern Illinois University, De Kalb, IL.

- The most common functional disorder of the gastrointestinal tract is**
 - ulcerative colitis
 - inflammatory bowel disease
 - irritable bowel syndrome
 - pouch ileitis
- Irritable bowel syndrome is a readily diagnosed disease because of the presence of many specific biomarkers in the patient's serum.**
TRUE FALSE
- Genetic factors may play a role in the development of inflammatory bowel disease.**
TRUE FALSE
- Crohn's disease (CD) is one type of**
 - ulcerative colitis
 - inflammatory bowel disease
 - irritable bowel syndrome
 - pouch ileitis
- Crohn's disease can involve many portions of the intestinal tract.**
TRUE FALSE
- A patient initially diagnosed as having ulcerative colitis may develop Crohn's disease over time due to extension of the disease.**
TRUE FALSE
- Antibodies to which of the following microorganisms have been noted in patients with Crohn's disease?**
 - bacteroides, listeria, Legionella, proteus*
 - Saccharomyces cerviciae, mycobacteria, E.coli, bacteroides*
 - Staphylococcus aureus, Campylobacter, Salmonella, enterococci*
 - Chlamydia trachomatis, Salmonella, Candida albicans, Giardia lamblia*
- The incidence of ulcerative colitis remains relatively stable but the frequency of Crohn's disease is increasing.**
TRUE FALSE
- The "ROME criteria," developed in an effort to standardize disease definition, are so-designated because they call for consideration of the patient's Rh blood group, obesity, mesocolic status, and results of an enterocentesis.**
TRUE FALSE
- Serum immune markers that have been identified as useful in the diagnosis and management of IBD include ANCAs — autoantibodies to cytoplasmic components of**
 - pancreatic acinar cells
 - neutrophils
 - vascular endothelium
 - hepatocytes
- Which of the following may be found in as many as 70 percent of Crohn's disease patients?**
 - Anti-OmpC
 - ANA
 - ANCA
 - ASCA
- Most patients with ulcerative colitis test positive for**
 - ANCA
 - ANA
 - ASCA
 - pancreatic antibodies
- The primary cytoplasmic antigen that triggers ANCA formation is**
 - elastase
 - cathepsin G
 - lactoferrin
 - serine proteinase 3
- Production of pANCA takes place in the**
 - salivary glands
 - gastric parietal cells
 - pancreatic acini
 - colonic mucosa
- Ulcerative colitis patients whose serum is positive for pANCA**
 - have a less aggressive form of the disease
 - are less likely to respond to treatment
 - seldom require surgical intervention
 - are at increased risk of hepatitis A

Continues on page 19

16. In IBD, the pANCA staining pattern is associated with antigens localized around the cell's
a. nucleoli
b. nucleus
c. mitochondria
d. lysosomes
17. Crohn's disease patients who are pANCA positive
a. have probably been misdiagnosed
b. have CD but present with clinical features of ulcerative colitis
c. are especially responsive to anti-TNF antibody therapy
d. tend to experience only one episode and then heal spontaneously
18. Ulcerative colitis predominantly affects the left side of the colon, while Crohn's disease tends to involve the right side of the colon and the ileum.
TRUE FALSE
19. The pANCA staining pattern has been noted in a high percentage of patients diagnosed with
1) autoimmune hepatitis
2) cirrhosis
3) primary sclerosing cholangitis
4) inflammatory bowel disease
a. 1 and 2
b. 2 and 4
c. 1, 2, and 4
d. 1, 3, and 4
20. Crohn's disease patients who are positive for both IgA and IgG ASCA seem to
a. have a more aggressive form of the disease
b. respond more readily to treatment
c. more frequently enter lengthy periods of remission
d. be at reduced risk for pancreatic involvement
21. pANCA that are DNase sensitive are typically seen in patients with
1) Crohn's disease
2) Ulcerative colitis
a. 1 and 2
b. 1 only
c. 2 only
d. neither 1 nor 2
22. The presence of pancreatic antibodies in children and adolescents is highly predictive of an especially aggressive form of Crohn's disease.
TRUE FALSE
23. Antibodies to an *E. coli* outer membrane porin antigen detected in the serum of a Crohn's disease patient would likely belong to which class of immunoglobulins?
a. IgA
b. IgG
c. IgM
d. IgE
24. Techniques that have proved useful in detecting serologic markers of IBD include all of the following EXCEPT
a. ELISA
b. indirect immunofluorescence
c. diagnostic imaging
d. DNase digestion of neutrophils
25. Sensitivity of ANCA assays have been reported to vary widely among laboratories.
TRUE FALSE
26. The positive predictive values for IBD of assays for ANCA and ASCA are not high enough to be clinically useful at the present time.
TRUE FALSE



TEST ANSWER FORM

SEROLOGIC MARKERS IN INFLAMMATORY BOWEL DISEASE — November 2001

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Antineutrophil Cytoplasmic Antibodies, Anti-*Saccharomyces cerevisiae* Antibodies, and Specific IgE to Food Allergens in Children with Inflammatory Bowel Diseases

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Differential diagnosis between ulcerative colitis (UC) and Crohn's disease (CD) is difficult in the initial phases in pediatric patients with inflammatory bowel diseases (IBD). This study was performed to determine the significance of anti-neutrophil cytoplasmic antibodies (ANCA) and anti-*Saccharomyces cerevisiae* antibodies (ASCA) in IBD. ANCA were specified with regard to their antigenic specificity, significance to the diagnosis, and correlation of titer with the disease activity. The occurrence of food allergy was questioned, too. Serum samples from 44 children with UC ($n = 23$) or CD ($n = 21$) and from disease-control children (coeliac disease, $n = 21$) were analyzed for IgG ANCA, ANCA target antigens, IgA and IgG ASCA, and IgE to food allergens. Results show that ANCA occur more frequently in UC than in CD and disease-control (74, 24, and 10%, respectively). The presence of ANCA does not reflect disease activity. Antigenic specificity does not differ in any group. IgA-ASCA are found more often in patients with CD (76% versus 17% in UC). The testing for both ANCA and ASCA enabled clear-cut differential diagnosis between UC and CD based on the high specificity (ANCA⁺ ASCA⁻ 92.5% for UC, ANCA⁻ ASCA⁺ 93.2% for CD). Specific IgE to food allergens were found in 8.7, 14.3, and 23.8% of patients with UC, CD, and coeliac disease, respectively. We conclude that combined testing of ANCA and ASCA represents a valuable tool in the differential diagnosis between UC and CD in pediatric patients, minimizing invasive diagnostic procedures. Monitoring of ANCA, its specificity, and titer determination does not bring more information. Testing for specific IgE to food allergens may be considered in individual patients. © 2002 Elsevier

Science (USA)

Key Words: anti-neutrophil cytoplasmic antibodies; anti-*Saccharomyces cerevisiae* antibodies; food allergy; inflammatory bowel diseases; children.

INTRODUCTION

Inflammatory bowel diseases (IBD)¹ represent a group of disorders of unknown etiology. Differential diagnosis between ulcerative colitis (UC) and Crohn's disease (CD) is difficult in the initial phases in pediatric patients with inflammatory bowel diseases. Non-organ-specific autoantibodies and other antibodies are associated with IBD, especially anti-neutrophil cytoplasmic antibodies (ANCA). ANCA are autoantibodies reacting with cytoplasmic antigens of polymorphonuclear leukocytes (PMN). Many ANCA target antigens have been identified, consisting mainly of components of primary granules of PMN, such as proteinase 3 (PR3), myeloperoxidase (MPO), lactoferrin (LT), bactericidal/permeability-increasing protein (BPI), and others (1–3). ANCA reacting with proteinase 3 are highly specific and sensitive diagnostic markers for primary vasculitis, Wegener's granulomatosis. Less specific are ANCA reacting with myeloperoxidase, which are found in another vasculitis, microscopic polyarteriitis, but also in other autoimmune inflammatory conditions (4–6). Besides the classic diagnostic association with vasculitides, ANCA have been shown to be a useful diagnostic marker for distinguishing between Crohn's disease and ulcerative colitis in adult patients (7–9), even if no specific ANCA target antigen is associated with these diseases. Due to the relative rarity of inflammatory bowel diseases during childhood, few studies deal with the association of ANCA in inflammatory bowel diseases in children (10–12); rarely is ANCA specificity analyzed in pediatric IBD.

Anti-*Saccharomyces cerevisiae* antibodies (ASCA) are antibodies reacting with mannans forming part of the microbial cell wall. They have been detected in patients with inflammatory bowel diseases, most often in those

¹ Abbreviations used: UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease; IIF, indirect immunofluorescence; PMN, polymorphonuclear leukocytes; PR3, proteinase 3; MPO, myeloperoxidase; BPI, bactericidal/permeability-increasing protein; LT, lactoferrin; ASCA, anti-*Saccharomyces cerevisiae* antibodies; ANCA, anti-neutrophil cytoplasmic antibodies.

suffering from Crohn's disease. Even if their pathogenic significance is not clear, they represent, alone or together with ANCA, a useful diagnostic marker in distinguishing Crohn's disease from ulcerative colitis in both children (13–15) and adults (16).

Intestinal inflammation is considered to be one of predisposing factors to food allergy (17). To our knowledge, no studies have focused on the complex analysis of pediatric patients suffering from IBD with respect to the occurrence of ANCA and its target antigens, ASCA, and specific IgE to food allergens.

PATIENTS AND METHODS

We examined 44 pediatric patients with IBD (23 UC, 21 CD, sex ratio (male/female) 14/7 UC, 11/12 CD, mean age 14.2 years for UC, 14.9 years for CD). Twenty-one patients with coeliac disease (control group) with the presence of anti-endomysial antibodies represented the control group. This group of patients was selected as a bowel disease control to exclude the possibility that the investigated parameters could be only a marker of bowel inflammation. The patients' characteristics are summarized in Table 1. Diagnosis of UC and CD was established according to clinical, radiological, endoscopic, and histological criteria.

ANCA were detected using indirect immunofluorescence of ethanol-fixed granulocytes. Antigenic specificity of ANCA against BPI, MPO, and PR3 was examined by ELISA anti-BPI (Binding Site, UK), anti-MPO, anti-PR3 (Binding Site, UK), and anti-lactoferrin (Euroimmun, Hamburg, Germany). Blood samples were collected repeatedly during the regular follow-up visits in an outpatient clinic during a 2-year period. ASCA in both isotypes IgA and IgG were examined by ELISA tests (Mediplan, Germany). IgE-specific antibodies to food allergens (chicken egg white, cow's milk, peanut, soy bean, apple, walnut, gliadin, carrot, and fish) were determined by ELISA-specific IgE (RISA, Germany).

The activity of disease was established according to clinical and laboratory criteria for pediatric patients (18). Patients were stratified into a highly active group, a moderate group, and a group in remission.

Fischer's exact test, the Mann-Whitney test, and a χ^2 test were used in statistical evaluation.

TABLE 1
Patient Characteristics

Diagnosis	n	Age	Mean age	Female	Male
UC	23	5–18	14.2	12	11
CD	21	12–18	14.9	7	14
Controls (coeliac disease)	21	2–18	11.3	13	5

TABLE 2
Statistical Significance of ANCA Positivity in IIF

Diagnosis	Total (n)	ANCA-positive (n)	ANCA-negative (n)
CD	21	5	16
UC	23	17	6
Control	21	2	19

Note. ANCA are found with a statistically higher frequency in patients suffering from ulcerative colitis than in those with Crohn's disease and than in control patients suffering from coeliac disease. UC vs CD, $P = 0.0022$; UC vs control, $P = 0.0021$.

RESULTS

ANCA in Indirect Immunofluorescence

Seventeen of 23 (74%) patients with UC and 5 of 21 (24%) patients with CD were P-ANCA-positive in indirect immunofluorescence (IIF) assay. The difference in the frequency of ANCA positivity in UC or CD was statistically highly significant (Table 2). In the longitudinal follow-up, ANCA positivity was a constant finding in individual patients (data not shown). No correlation between the presence of ANCA and disease severity or activity was found (Table 3).

ANCA Specificity

Ulcerative colitis. None of the patients with UC reacted with PR3, five patients had antibodies reacting with MPO (29.4%) and four with BPI (23.5% of positive patients), and two reacted with lactoferrin (11.8% of positive patients) (Table 4). Nine of the P-ANCA-positive patients did not react with any of the tested antigens. Sera of three patients showed a positive reaction with two target antigens (BPI, LT).

Crohn's disease. In CD group of patients, three reacted with BPI, seven with MPO, and none with lactoferrin and PR3 (Table 4). One of the ANCA-positive patients reacted with none of these antigens. Sera of two patients showed a positive reaction with two target antigens (MPO, BPI).

TABLE 3
Correlation between ANCA and Disease Activity

Diagnosis	ANCA (IIF)	High activity (n)	Moderate activity (n)	Remission (n)
CD	+	3	1	1
	–	9	2	5
UC	+	3	7	7
	–	0	4	2

Note. The presence of ANCA does not reflect the disease activity in both groups of patients. CD ANCA⁺ vs CD ANCA[–], $P = 0.73$, not significant (n.s.); UC ANCA⁺ vs UC ANCA[–], $P = 0.75$, n.s.

TABLE 4
Correlation between Antigen Specificity of ANCA and Diagnosis

ELISA	Anti-PR3			Anti-MPO			Anti-BPI			Anti-LT		
	+	-	n	+	-	n	+	-	n	+	-	n
CD	0	21	21	7	12	19	3	16	19	0	18	18
UC	0	23	23	5	18	23	4	18	22	2	20	22
P value				P = 0.32, n.s.			P = 1.0, n.s.			P = 0.49, n.s.		

Note. The number of patients reacting with MPO, BPI, or LT does not differ between both groups of patients. Anti-PR3 antibodies were not detected in any patient. +, positive result; -, negative result; n, total number of patients; n.s., not significant.

Titer of Antibodies to Specific Antigens in the Correlation with Diagnosis

Proteinase 3. No patients with UC or CD reacted with PR3.

Lactoferrin. Lactoferrin as a target antigen of ANCA was not found in children with CD and was positive in two children with UC (Table 5). One girl with an extreme titer of anti-lactoferrin ANCA had a severe clinical course of UC (data not shown).

Bactericidal/permeability-increasing protein. Anti-BPI antibodies were found positive in 4 of 17 of patients with UC (1 boy had a very high titer of anti-BPI concomitantly with the positivity of MPO). Three patients with CD were reacting with BPI. The titer tended to be higher in UC patients, but this trend did not reach statistical significance.

Myeloperoxidase. Anti-MPO antibodies were found in five other patients with UC, in seven patients with CD, and even in two patients who were negative in the indirect immunofluorescence test. The titer was higher in CD patients than in UC patients with statistical significance.

Anti-Saccharomyces cerevisiae Antibodies

ASCA of the IgA isotype were found in 16 patients with CD (76%); in 8 children of this group the titer of

ASCA was very high and the ASCA of the IgG isotype tested positive in 8 patients (38%). Seven patients were ASCA-positive in both the IgG and IgA isotypes. In the UC group, only 4 patients reacted with *Saccharomyces cerevisiae* antigens (3× borderline titer and 1× moderately elevated) (17.7%) (Table 6). The presence of ASCA did not correlate with disease activity (Table 7). In coeliac patients, 4 (19%) were found ASCA IgA-positive and 1 of them (4.8%) reacted with both IgG and IgA isotypes. The titer of ASCA was significantly higher in patients with Crohn's disease than in those with coeliac disease (CD, mean = 124.0 U/ml, median = 26.3 U/ml, SD = 210.4; coeliac disease, mean = 17.6 U/ml, median = 13.4 U/ml, SD = 10.4, $P = 0.0012$).

ASCA and ANCA

In the CD group, two patients showed positive P-ANCA in the IIF test and positivity for both ASCA IgG and ASCA IgA. Another two patients were positive in both P-ANCA and ASCA IgA. In the UC group, only one patient was positive for both P-ANCA and ASCA IgA and IgG. In the coeliac group, three patients showed positivity for ASCA IgA and one patient for ASCA IgA and IgG. Another two patients were positive for P-ANCA in the IIF test. Table 8 shows the specificity and sensitivity of ANCA and ASCA testing (separate and combined investigation). ANCA positivity alone was rather characteristic for patients with UC

TABLE 5
Correlation between the Titer of Specific ANCA Autoantibodies and Diagnosis

Diagnosis	n	Anti-BPI titer			Anti-MPO titer			Anti-LT titer		
		+	±	U/ml	+	±	U/ml	+	±	RU/ml
CD	21	3	0	10.3	4	3	10.1	0	0	3.3
UC	23	3	1	11.6	2	3	4.6	2	0	14.5

Note. The titers of antibodies reacting with bactericidal/permeability-increasing protein and lactoferrin did not differ in either group of patients. The titer of anti-myeloperoxidase antibodies was higher in patients with Crohn's disease. +, number of patients with high titer; ±, number of patients with borderline value; U/ml, RU/ml, mean value in the appropriate ELISA in units or relative units, respectively. Anti-MPO, CD vs UC, $P = 0.0083$; very significant; Anti-BPI, CD vs UC, $P = 0.3696$, (n.s.); Anti-LT, CD vs UC, $P = 0.8895$, n.s.

TABLE 6
ASCA in IBD Patients

Diagnosis	Total	ASCA IgA		ASCA IgG	
		+	-	+	-
CD	21	16	5	8	13
UC	23	3	20	2	21
Coeliac disease	21	4	17	1	20

Note. Anti-*Saccharomyces cerevisiae* antibodies of both IgA and IgG isotypes are most often found in patients suffering from Crohn's disease. This difference is highly statistically significant, especially in the case of IgA ASCA. ASCA IgA, CD vs UC, $P < 0.0001$, very significant (v.s.); ASCA IgG, CD vs UC, $P = 0.031$, significant (s.); ASCA IgA, CD vs coeliac disease, $P = 0.0002$, v.s.; ASCA IgG, CD vs coeliac disease, $P = 0.02$, s.; ASCA IgA, UC vs coeliac disease, $P = 0.069$, not significant (n.s.); ASCA IgG, UC vs coeliac disease, $P = 1$, n.s.

(specificity 0.83, sensitivity 0.74). ASCA IgA positivity was typical for CD patients with a specificity of 0.84 and a sensitivity of 0.76. For ASCA IgG, specificity was high (93%), but sensitivity was low due to a small sample of patients (0.38). The combination ANCA⁺ ASCA⁻ is specific (specificity, 0.93) for UC patients, with a sensitivity of 0.61. ANCA negativity and positivity for both IgG and IgA ASCA is 98% specific for CD, with low sensitivity (24%).

Specific IgE to Food Antigens

Specific IgE to food allergens in CD, UC, and coeliac control patients is shown in Table 9.

Ulcerative colitis. Two patients (8.7%) with UC had specific IgE antibodies. One was positive in IgE specific for carrot and gliadin (RAST class 2), a second had a borderline titer of anti-carrot, anti-gliadin, and anti-soya IgE (RAST class 1). Twenty-one patients were negative for specific IgE.

Crohn's disease. Three patients (14.3%) with CD had specific IgE. One reacted with chicken egg white

TABLE 7
Correlation between ASCA and CD Activity

Diagnosis	ASCA IgA	ASCA IgG	High activity (n)	Moderate activity (n)	Remission (n)
CD	+	+	5	1	1
CD	+	-	6	1	2
CD	-	+	0	0	1
CD	-	-	2	1	1

Note. No correlation between the presence of anti-*Saccharomyces cerevisiae* antibodies and the disease activity was found. CD ASCA⁺ vs CD ASCA⁻, $P = 0.2621$, not significant.

TABLE 8
Antibodies, ANCA and ASCA in IBD

	CD	UC	Coeliac disease	Sensitivity (%)	Specificity (%)
ANCA ⁺	5	17	2	74	83 ^a
ASCA IgA ⁺	16	3	4	76.2	84 ^b
ASCA IgG ⁺	8	2	1	38	93.2 ^b
ANCA ⁺ , ASCA IgA ⁻ , ASCA IgG ⁻	1	14	2	60.9	92.5 ^a
ANCA ⁻ , ASCA IgA ⁺ , ASCA IgG ⁺	5	0	1	24	97.7 ^b
ANCA ⁻ , ASCA IgA ⁺	6	0	3	28.6	93.2 ^b

Note. The presence of ANCA was more typical for patients with ulcerative colitis, with a specificity of 83% and sensitivity of 74%. The presence of ASCA in the IgA isotype was typical for patients with Crohn's disease, with a specificity of 84% and sensitivity of 76.2%. Combined serological testing for both ANCA and ASCA increased the specificity up to 93.2% in the case of ANCA-negative, ASCA IgA-positive for Crohn's disease (with a sensitivity of 28.6%), and a specificity of 92.5% in the case of ANCA positivity, ASCA negativity (with a sensitivity of 61%) for ulcerative colitis.

^a Specificity for UC.

^b Specificity for CD.

(RAST class 2) and carrot (borderline value, RAST class 1). The sera of a second reacted with gliadin (RAST class 2), and third patient had borderline titers for (RAST class 1) of soy, carrot, and chicken egg white. All other patients (18) tested negative for specific IgE.

Controls (coeliac). Among coeliac patients, five (23.8%) had IgE specific to one or more of six food allergens (chicken egg white, apple, and walnut, RAST class 2; soy bean and peanut, RAST class 1; and carrot, RAST class 3), two patients were positive for chicken egg white (RAST classes 1 and 2), and another two were positive for milk (RAST classes 1 and 2).

The differences between the three groups in the occurrence of IgE positivity were not statistically significant.

DISCUSSION

Ulcerative colitis and Crohn's disease represent a group of bowel inflammation of unknown etiology. Many components of the immune system play a role in the pathogenesis of the gut tissue damage (19, 20). Immune dysfunction may be involved in the etiology of the diseases, as experimental animals ("knock-out") with disrupted genes encoding several important molecules of the immune system, like IL-2 or IL-10, develop, under microbial challenge, clinical conditions resembling human CD (21, 22). The contribution of ANCA to the pathogenesis of IBD is unclear. ANCA have been shown to play a substantial role in other ANCA-associated diseases (5, 6, 23). ANCA influence neutrophil function leading to an increase of oxidative

TABLE 9
Specific IgE Antibody to Food Allergens in Patients

Diagnosis	<i>n</i>	Chicken egg white	Cow's milk	Peanut	Soy bean	Apple	Walnut	Glutadin	Carrot	Fish
CD	21	2*	0	0	1	0	0	1	2	0
UC	23	0	0	0	1	0	0	2	2	0
Control (coeliac disease)	21	3	2	1	1	1	1	0	1	0

Note. Specific IgE to food allergens were found in 8.7, 14.3, and 23.8% of patients with UC, CD, and coeliac disease, respectively. The difference in the frequency of IgE positivity is not statistically significant among the groups. *n*, total number of patients.

* Number of patients positive for food allergens.

metabolism (24–26). In our previous studies, we proved that anti-PR3, anti-MPO, and anti-BPI-positive sera inhibit the microbicidal activity of PMN (27–29). Analogously, we suspect that inhibition of the antimicrobial activity of PMN by ANCA in IBD may influence the balance between pathogenic and physiological intestinal flora. A break of the tolerance to intestinal bacteria is supposed to be the main mechanism in the pathogenesis of IBD (30, 31), apart from, or together with, a genetic background (32, 33).

In our group of pediatric patients with IBD, the presence of ANCA was detected more frequently in UC patients than in CD patients, which is in accordance with the results of others (10, 11). The titer of autoantibodies did not correlate with the diagnosis. As was previously shown, ANCA positivity did not reflect disease severity or activity, which, according to our results, is true as well for ANCA target antigen specificity. This was already documented for adult patients (reviewed in 34). ANCA positivity is a very stable finding, as ANCA-positive patients stay positive during long-term follow-up periods. ANCA are not only an epiphenomenon of chronic intestinal inflammation, as our control group of coeliac patients showed a statistically significant lower frequency of ANCA.

The presence of antibodies reacting with more than one antigen was already documented in several studies in adult patients (35–38). We can confirm that the same is true for children. ANCA target specificity is not exclusively connected with UC or CD, and antibodies reacting with any of the particular tested autoantigens were found in both groups of patients.

Anti-*Saccharomyces cerevisiae* antibodies were frequently positive in patients with Crohn's disease, which is in accordance with the results of others (13–16). Its contribution to the differential diagnosis of CD versus UC is stressed, especially in combined serological testing for both parameters. The sensitivity and specificity reached in our study are comparable to findings already published (13, 15, 16). The sensitivity was not high enough for population testing, but the speci-

ficity values reflect the clear clinical importance of ANCA and ASCA evaluation in the children with IBD.

Food allergy is one factor considered to play a role in IBD (17). In one study, specific IgE to food allergens was not found in patients with Crohn's disease (39). In our study, 8.7% of UC patients, 14.3% of CD patients, and 23.8% of coeliac patients had detectable specific IgE to different food antigens. The differences between the three groups were not statistically significant. Even if the true prevalence of food allergy in childhood is not known due to several factors, particularly the problems in appropriate diagnostic testing, the estimated prevalence is about 2% in the general population and 4–6% in children (40). The finding of specific IgE in all groups of patients may reflect more the propensity for mast cells in inflamed mucosa to produce IL-5 (41) than the etiologic association between IBD and food allergy. However, the ratio of patients having specific IgE to several food allergens in both groups is not negligible and, at the individual level, elimination in the diet may be considered in these patients.

As we still do not understand all the subtle mechanisms of maintaining the balance between tolerance and immunity to dietary and bacterial antigens of the gut, nor what is the trigger for ANCA, ASCA, and specific IgE production, we cannot explain the precise relationship among these parameters in childhood IBD. However, our study confirms that combined serological testing for ANCA and ASCA may be helpful in distinguishing UC and CD in children suspected of having inflammatory bowel disease, thus minimizing invasive diagnostic procedures. The contribution of elimination in the diet to the disease course in patients having specific IgE antibodies to food allergens requires further studies.

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Inflammatory Bowel Disease in Children 5 Years of Age and Younger

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OBJECTIVES: Clinicians are becoming increasingly aware that inflammatory bowel disease (IBD) can affect all age groups, although it has not been well described in infants and young children. Our aim was to evaluate early onset IBD in patients 5 yr of age and younger.

METHODS: Medical records of patients diagnosed with early onset IBD at The Children's Hospital of Philadelphia between 1977 and 2000 were reviewed. Patients were divided into three categories: those with Crohn's disease (CD), those with ulcerative colitis (UC), and those with indeterminate colitis (IC).

RESULTS: A total of 82 patients fulfilled the criteria. In 12 patients (15%), the IBD diagnosis was changed during the course of illness. At the end of the follow-up period, linear growth failure was present in 10 of 35 (29%) children with CD, one of 30 (3%) with UC, and three of 17 (18%) with IC. Failure to thrive was a frequent presenting symptom in children with CD (44%) and IC (39%), whereas in all four patients with UC and failure to thrive the diagnosis was subsequently changed to CD or IC. A high proportion of patients with CD had large bowel (89%), and perianal (34%) disease. None of the tested patients were positive for anti-*Saccharomyces cerevisiae* antibody (ASCA), and 10 tested positive for perinuclear antineutrophil cytoplasmic antibody (three of five patients with CD, five of seven with UC, and two of three with IC).

CONCLUSIONS: Failure to thrive, at the time of presentation, is indicative of a final diagnosis of CD or IC, not UC. Linear growth failure is a common finding in patients with early onset CD. A high proportion of patients with CD have failure to thrive, colonic, and perianal disease. The IBD serology panel is of limited clinical relevance in providing definitive diagnostic information in this pediatric population. (Am J Gastroenterol 2002;97:2005–2010. © 2002 by Am. Coll. of Gastroenterology)

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory bowel diseases of unclear etiology. The prevailing hypothesis regarding the pathogenesis of

inflammatory bowel disease (IBD) combines both environmental factors and an altered immune response in genetically predisposed patients, which then leads to chronic inflammation of the intestinal tract (1). Recently, a mutation in the gene known as Nod2, which resides on chromosome 16 and encodes the protein recognizing lipopolysaccharides (LPS) of the bacterial outer cell wall membrane, has been identified twice as frequently in patients with CD as in the general population (2, 3). In patients with CD, the inability to recognize LPS may lead to an exaggerated inflammatory response without the innate immune system modulation. Living in a more sterile environment has been shown to delay exposure to enteric infections in early life, possibly resulting in failure of the normal maturation process necessary to develop normal intestinal tolerance (4–6).

During the last several decades, the incidence of IBD in adults is increasing (7). Pediatric epidemiological studies indicate the same patterns of increased incidence for both types of IBD (8–11). The reasons for this increase are unclear, and the contributing factors may occur early in life. Very young patients with IBD require special attention because of the potentially large impact of the disease on their growth and development.

The aim of the present study was to describe the presenting signs and symptoms along with the disease progression in children 5 yr of age and younger with early onset inflammatory bowel disease (EO-IBD). This information may help to improve care for children who present with IBD at this early age.

MATERIALS AND METHODS

A retrospective analysis of the database with all IBD patients followed at the Children's Hospital of Philadelphia between 1977 and 2000 was performed. A total of 94 patients diagnosed with EO-IBD were identified. Twelve patients were excluded from the study: three did not meet the histological criteria for IBD after review by one pathologist (P.R.), and medical records were incomplete for six patients and were not available for three. Among the six patients with incomplete records whose diagnoses could not be confirmed, four carried the diagnosis of UC and two of

CD. In all, 82 patients were included in the study. The diagnoses were confirmed by standard endoscopic, histological, and radiographic criteria (12). The patients were divided into three categories: those with CD, those with UC, and those with indeterminant colitis (IC). The diagnosis of IC was assigned to patients with chronic inflammation limited to the large intestine for whom (on the basis of clinical, laboratory, radiological, endoscopic, or histological criteria) it was not possible to distinguish between CD and UC. All patients had colonoscopy or flexible sigmoidoscopy performed at the time of diagnosis, and all but one patient had a radiographic study during the course of the disease (upper GI series with a small bowel follow-through, or barium enema). The remaining patient had the diagnosis made at the time of surgery. The medical records and x-ray reports were reviewed by the two investigators (P.M. and G.T.) and by the patients' primary gastroenterologists. The diagnosis of the small intestinal CD was made based on the radiographic study.

The following characteristics were investigated: age, sex, diagnoses and change in diagnoses over time, length of follow-up, presenting symptoms, diagnostic time lag, medications, surgical procedures, anatomic location of the disease, growth data, family history, and results of anti-*Saccharomyces cerevisiae* antibody (ASCA) and perinuclear antineutrophil cytoplasmic antibody (p-ANCA) testing. The family history was defined as a history of IBD in parents, grandparents, aunts or uncles, and first cousins. Linear growth failure was defined as height below the fifth percentile on a height for age growth curve developed by the National Center for Health Statistics in 1979, and failure to thrive (FTT) as weight below the fifth percentile on a weight for age growth curve. Measurements of linear growth were obtained at the end of the follow-up period. Weight measurements for definition of failure to thrive were recorded at the time of diagnosis. Perianal disease was defined as perianal fistula or perianal abscess.

The association between presenting symptoms and initial diagnoses were evaluated using univariate analysis with the χ^2 test. Statistical analysis was performed using Stata version 6.0 software (Stata, College Station, TX). This study was approved by the Institutional Review Board of the Children's Hospital of Philadelphia.

RESULTS

A total of 82 patients (47 male and 35 female; sex ratio, 1.34) were diagnosed with EO-IBD. The median follow-up was 7.5 yr (range, 6 months to 23 yr). Initially, 36 children (44%) were diagnosed with UC, 27 (33%) with CD, and 19 (23%) with IC.

Figure 1 shows the changes in diagnoses. The majority of patients (nine of 12) in whom the diagnosis was changed had the initial diagnosis made before 1991. The age distribution and final diagnoses are shown in Figure 2. Only one

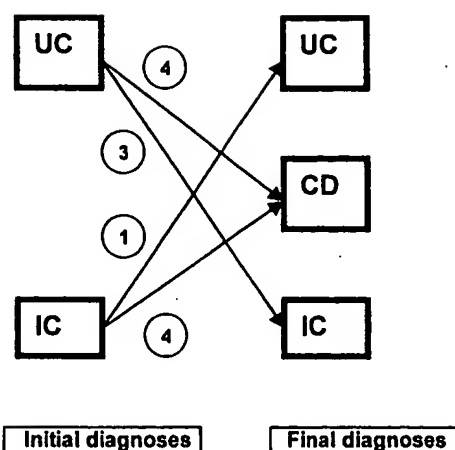


Figure 1. Changes in diagnoses among study patients over time.

of 19 patients (6%) diagnosed with EO-IBD below the age of 2 yr was diagnosed with UC.

In all, 23% of patients with CD and UC and 18% of patients with IC had a family history of IBD. When 13 patients whose family history was not available were excluded, 28% of patients with CD and UC and 20% of patients with IC had a family history of IBD. The age of onset of disease in relatives was not available.

Linear growth failure was present in 10 of 35 children (29%) with CD, one of 30 (3%) with UC, and three of 17 (18%) with IC at the end of the follow-up period.

At the time of initial diagnosis, 26% of patients with CD had inflammation in the stomach, 22% in the duodenum, 19% in the small bowel, 52% in the terminal ileum, and 89% in the large bowel. In all, 34% had perianal disease. Of the CD patients, 60% had nonstricturing nonpenetrating (inflammatory) type of the disease, 34% penetrating (fistulizing), and 6% stricturing type. Of the patients with UC, 60% had isolated left-sided colitis, and 40% had pancolitis.

Presenting symptoms are shown in Table 1. Blood in the stool (hematochezia) was more commonly associated with UC than with IC and CD combined ($p = 0.0002$). Failure to thrive at the time of initial presentation was more common in patients with CD or IC than in those with UC ($p = 0.004$). All four patients initially diagnosed with UC who had FTT as a presenting symptom had their diagnosis changed to IC or CD ($p < 0.0001$). Chronic fever was associated with CD and not with UC or IC ($p = 0.015$). Vomiting was associated with CD or IC but not UC ($p = 0.01$). The median diagnostic lag (*i.e.*, median time between onset of symptoms and time of diagnosis) was 4.5 months for patients with CD, 2 months for UC, and 6.5 months for IC.

Serological testing for pANCA and ASCA was performed in 15 children before the age of 6 yr. All samples were tested by the Prometheus Laboratories (San Diego, CA). None of the patients had positive ASCA test results, but 10 patients had positive results for pANCA. Three of five patients (60%) with CD, five of seven (71%) with UC,

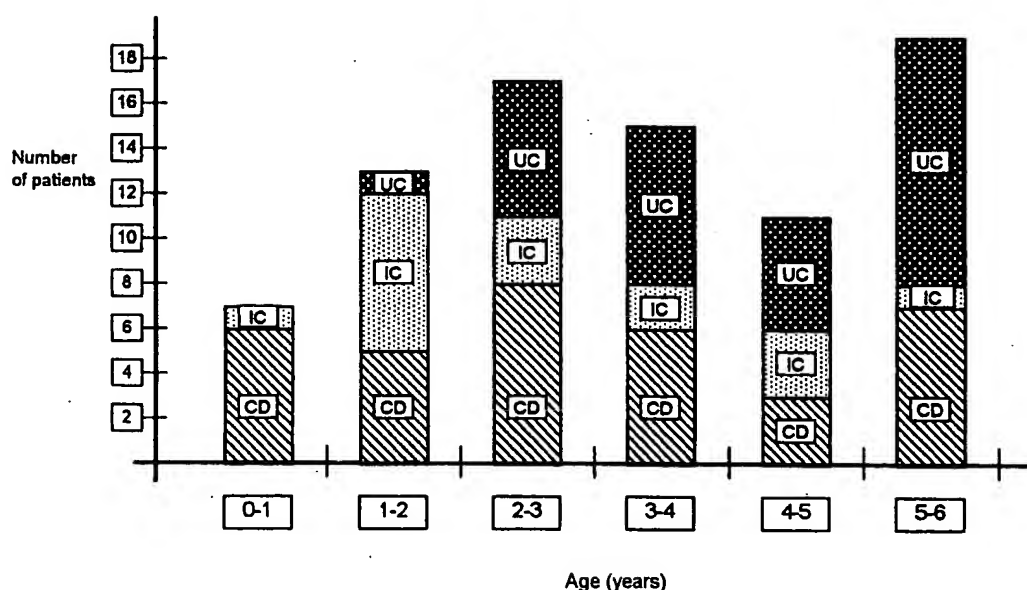


Figure 2. Age at time of diagnosis and final diagnosis.

and two of three (66%) with IC had positive pANCA results. The presence of pANCA antibodies was not significantly more prevalent in UC than in CD or IC.

Corticosteroids were used before the age of 6 yr in 67% of patients with CD, 67% of patients with UC, and 52.6% of patients with IC. 6-Mercaptopurine was used before the age of 6 yr in 22%, 11%, and 26% of patients with CD, UC, and IC, respectively.

During the course of their disease, 10 patients (12%) required surgical intervention: five patients (14%) with CD, four (13%) with UC, and one (6%) with IC. The patient with IC underwent colectomy because of toxic megacolon. Three patients with CD had resection of a stricture, one had a diverting colostomy secondary to severe perianal disease and stricture, and one had perforation requiring partial colectomy and recto-vaginal fistula repair. Four patients with UC had a colectomy performed: one for fulminant colitis unresponsive to *i.v.* corticosteroids and cyclosporin, and three for severe disease with failure of chronic immunomodulatory therapy.

DISCUSSION

Reports of young children with IBD have been published for several decades (13–17). Most case series have involved children of different ages, and only a few have concentrated exclusively on young children (18, 19). Recently, evidence for increased incidence of IBD not only in adults but also in children and adolescents has caused renewed interest in the younger age group (19). So far, this is the largest study of children aged 5 yr and younger who have been diagnosed with EO-IBD.

The genetic factors in the etiology of IBD are well recognized, with a high rate of concordance between monozygotic twins (44.4%) compared with dizygotic twins (3.8%) among patients with CD (20). A recent report described multiple siblings affected with CD (21). In our series, 28% of patients with CD had a family history of IBD. This correlates with the data from a combined adult and pediatric study, in which 29.9% of patients diagnosed with CD before age 20 yr had a positive family history compared with only 13.6% of patients whose diagnoses were made at a later age

Table 1. Presenting Symptoms of Study Patients

Symptom	CD (n = 27)	UC (n = 36)	IC (n = 18)
Diarrhea	81%	79%	89%
Blood in the stool*	67%	94%	67%
Abdominal pain	67%	33%	33%
Failure to thrive*	44%	11%	39%
Perianal disease	34%	0%	0%
Vomiting*	15%	0%	22%
Constipation	4%	0%	6%
Chronic fever*	11%	0%	0%

**p* < 0.05.

(22). Genetic anticipation, which was described in parent-child pairs in Huntington's disease (23), may explain this finding. Genetic anticipation is a concept whereby the affected offspring manifest the disease at an earlier age, and sometimes in a more severe form, than does the affected parent. In the future, it will be of interest to investigate how many children with EO-IBD are positive for the recently discovered gene *Nod2* associated with CD (2).

Our finding of only one patient diagnosed with UC within the first 2 yr of life differs from the report by Gryboski, in which UC was found to be more common than CD at this age (19). The reason is unclear. Of the patients with CD, 60% were diagnosed during the period between 1991–2000 in our study, whereas the study period in Gryboski's report ended in 1990. It is possible that changes in the epidemiology of the disease, as well as changes in the practice of performing full colonoscopies as opposed to flexible sigmoidoscopies, thus allowing histological sampling of the terminal ileum, contributed to the difference.

A striking number of patients with CD (29%) were noted to have linear growth failure at the final follow-up, indicating that despite early diagnosis and treatment, a significant proportion of patients are not able to achieve appropriate growth. Previous studies have reported growth failure in 7–30% of patients with CD (24). In a study by Kanof *et al.* (25), 25% of patients with CD developed severe linear growth failure (height below the fifth percentile), and in a study by McCaffery *et al.* (26), 18% of patients with IBD demonstrated height below the third percentile. Growth failure most likely occurs because of a combination of several detrimental factors: malnutrition, the effects of inflammatory cytokines, and iatrogenic causes (*e.g.*, corticosteroid therapy). Nutritional therapy has previously been shown to be beneficial in improving growth (27). However, nine of 10 patients with CD and linear growth failure in this group received either nasogastric or gastric tube feeding during the course of their illness. A new form of biological therapy directed against tumor necrosis factor- α with a potential for improved histological healing may have a beneficial effect on the final growth in patients with CD (28–32).

The anatomic distribution of the disease in this group of patients with CD is different from that in previously published studies in older children and adolescents (33). In our study, isolated small bowel disease was seen in only 11% of patients, isolated large bowel disease in 30%, and small and large bowel disease in 59%, resulting in a total of 89% of patients with large bowel disease. Pooled data of 14 pediatric studies involving a total of 1153 older children revealed that only 58% of patients had large bowel disease (34). Another study reported that patients younger than 20 yr were more likely to have small intestinal disease when compared with patients 40 yr and older (22). The reasons for the noted difference are unclear. However, patients with CD diagnosed at a very early age may represent a new subgroup with a distinct anatomic disease distribution.

Presenting symptoms can be helpful in differentiating between CD and UC. Hematochezia was more common in UC than in CD or IC. Vomiting was associated with CD and IC, and chronic fever was associated exclusively with CD. Most importantly, in this cohort with early onset CD and IC, failure to thrive (FTT) as a presenting symptom was present in 44% and 39% of patients, respectively. Four patients (12%) initially diagnosed with UC had FTT as a presenting symptom. Interestingly, all four patients had their diagnosis subsequently changed from UC to IC or CD. One of these patients was subsequently found to have granulomas in the large intestine, one had severe inflammation of the terminal ileum on a repeat colonoscopy, one had persistent inflammation of the ileum after the colectomy, and one was found to have patchy inflammation on subsequent colonoscopies. At the same time, none of the remaining 32 patients initially diagnosed with UC had FTT. We therefore conclude that failure to thrive is a presenting symptom that is predictive of CD or IC and not UC. The only other available study that examined FTT as one of the symptoms of IBD (33) described it in 25% of patients with CD, which is less than in our series, and in none in UC, which corresponds to our results. This may possibly be due to more severe disease at the time of diagnosis in our group of patients with CD.

More than 20% of patients with EO-IBD had the diagnosis of indeterminant colitis. In a large, multicenter study of European adults, 5% of newly diagnosed patients were diagnosed with IC (35). The large proportion of children with large bowel involvement could possibly explain this difference. In pediatric series of older children, 14–23% were diagnosed with IC (8), which is similar to our results. Changes in diagnoses occurred more frequently in patients whose diagnoses were made before 1990. This could be explained both by the longer duration of follow-up, allowing the establishment of correct diagnosis, or by improvements in the technical aspects of pediatric colonoscopy during the last decade, allowing better visualization and tissue sampling of the terminal ileum.

Serological assay is a potentially important addition to the diagnostic armamentarium in IBD. Combined measurement of pANCA and ASCA has been advocated as a valuable diagnostic approach in older children and adults. The combination of positive pANCA and negative ASCA had 57% sensitivity and 97% specificity for the diagnosis of UC, and the combination of positive ASCA and negative pANCA had 47% sensitivity and 97% specificity for the diagnosis of CD (36). No study with long term follow-up has been performed to validate the use of these tests to differentiate between UC and CD in a setting of indeterminate colitis (37). In a study of older children, Ruemmele *et al.* reported that the combined test had 57% sensitivity and 92% specificity for UC, and 55% sensitivity and 95% specificity for CD (38). None of the patients with EO-IBD in our study had a positive ASCA. One can postulate that several years of exposure to *Saccharomyces cerevisiae* in an individual with increased intestinal permeability are necessary to produce

detectable ASCA levels (38). The percentage of positive pANCA was similar among UC and CD patients. Because only 15 patients had testing performed before the age of 6 yr, the sample size may be too small to draw reliable conclusions. A larger study is necessary to confirm these important findings.

Finally, surgery was performed in 12.2% of patients during the course of their disease, a higher proportion than the 5% of patients reported in a pediatric series of patients under 10 yr of age (19). Another study of Crohn's disease in children reported 50% and 70% of patients requiring surgery within the first 10 and 15 yr of diagnosis, respectively (39). Among adult patients with UC, almost one half will undergo surgery within the first 10 yr of their illness, and more than 75% of patients with CD will have surgery during the first 20 yr of the disease (40). Length of the follow-up is most likely the reason for the small proportion of children requiring surgery in our series. Continued follow-up may provide further information. Also, a small number of patients in this group were found to have small intestinal CD, which may be associated with a greater need for surgery because of stricturing disease.

In summary, we describe a unique subgroup of young patients with EO-IBD. Accurate differentiation between CD and UC in this age group is very difficult. We noted a high proportion of patients with Crohn's disease with linear growth failure and large bowel disease. Failure to thrive at the time of presentation was predictive of a final diagnosis of CD or IC and not UC. The combined testing of pANCA and ASCA is of limited clinical use for differentiating between CD and UC in children 5 yr of age or younger.

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Antigen specificity of circulating anti-neutrophil cytoplasmic antibodies in inflammatory bowel disease.

Kossa K, Coulthart A, Ives CT, Pusey CD, Hodgson HJ.

Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London, UK.

OBJECTIVES: To characterize the antigen specificity of circulating anti-neutrophil cytoplasmic antibodies (ANCAs) in inflammatory bowel disease (IBD). DESIGN: Analysis of the prevalence of circulating ANCAs in patients with ulcerative colitis and Crohn's disease, by both non-specific methods (immunofluorescence against fixed neutrophil leukocytes) and specific antigen techniques (against purified neutrophil leukocyte constituents). METHODS: Indirect immunofluorescence against fixed polymorphonuclear leukocytes, and solid-phase enzyme-linked immunosorbent assay (ELISA) against neutrophil constituents (alpha-granules, elastase, myeloperoxidase, cathepsin g, lysozyme and lactoferrin). RESULTS: Although results using immunofluorescence were typical of other studies (ulcerative colitis positive in 41%, Crohn's disease in 10%), ELISA studies showed antibody activity against neutrophil components in 69% of patients with ulcerative colitis and 39% of those with Crohn's disease. Antibodies in ulcerative colitis were commonly directed (in descending order) against lysozyme, cathepsin G, elastase, and lactoferrin, and in Crohn's disease against lysozyme. CONCLUSION: Correlation of indirect immunofluorescence data and ELISA results indicated that even this large panel of specific antigens fails to identify all the ANCA targets in IBD. The lack of correlation between the findings of ANCAs, either in general or versus a specific target, and disease extent or activity in ulcerative colitis supports the suggestion that ANCAs are unlikely to be of primary importance in pathogenesis.

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Konrad Kossa, Anne Coulthart, Carole T. Ives,
Charles D. Pusey and Humphrey J.F. Hodgson

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Design: Analysis of the prevalence of circulating ANCA in patients with ulcerative colitis and Crohn's disease, by both non-specific methods (immunofluorescence against fixed neutrophil leukocytes) and specific antigen techniques (against purified neutrophil leukocyte constituents).

Methods: Indirect immunofluorescence against fixed polymorphonuclear leukocytes, and solid-phase enzyme-linked immunosorbent assay (ELISA) against neutrophil constituents (α -granules, elastase, myeloperoxidase, cathepsin G, lysozyme and lactoferrin).

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Keywords: anti-neutrophil cytoplasmic antibodies, ulcerative colitis, Crohn's disease, elastase, lactoferrin, lysozyme, cathepsin G

Introduction

Circulating anti-neutrophil cytoplasmic antibodies (ANCA) were first recognized in systemic vasculitis, and in particular in Wegener's granulomatosis [1]. Since this report, the spectrum of disease in which circulating ANCA has been described has dramatically widened. Apart from a variety of vasculitic and rheumatological conditions [2,3], ANCA have been strongly associated with primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD), specifically ulcerative colitis

[4-8], and less strongly with a number of infections and neoplasms [9].

The technique used initially for identifying ANCA was indirect immunofluorescence, and a number of different ANCA staining patterns on polymorphonuclear leukocytes (PMNLs) has been described. The best defined are cytoplasmic ANCA (cANCA) and perinuclear ANCA (pANCA), but in addition others have recognized a pattern different from these antibodies termed atypical ANCA (xANCA), particularly in

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non-vasculitic conditions [9]. It is clear that there are a number of distinct intracellular antigens recognized by ANCAs and that the precise staining patterns reflect antigen specificity of the circulating antibodies, together with technical factors such as fixation methods, which may result in destruction or relocation of antigens within the cell [10].

The target antigens identified are intracellular enzymes. Most of these are predominantly localized within cytoplasmic granules of PMNLs — conventional histochemistry identifies two predominant granule types: primary or α -granules, in which the enzymes elastase, myeloperoxidase, cathepsin G and proteinase 3 are localized, and the specific or β -granules, in which lactoferrin and alkaline phosphatase are present. However, the segregation of enzymes into granules judged in this way does not seem to be absolute, and, for example, lysozyme is identified in both these granule forms [11].

There are conflicting results concerning the patterns of ANCA in IBD; p-, c- and xANCAs have been previously described [6], and there are a number of reports that different enzymes such as cathepsin G [12] and lactoferrin [13], but not proteinase 3 [8], appear as specific targets. In this study we analysed the sera of patients with ulcerative colitis, Crohn's disease, and a selected group of vasculitis patients for the presence of ANCAs against a panel of specific antigens by solid-phase enzyme-linked immunosorbent assays (ELISA), using target antigens characteristic of both α and β -granules. We investigated the relationship between the immunofluorescence ANCA pattern and specific antibody levels, and the relationship between ANCAs and disease distribution, activity and therapy.

Patients and methods

Sera were taken from the following groups of patients and stored at -20°C until use.

Ulcerative colitis

Forty-nine patients with ulcerative colitis were studied: 24 men aged 27–80 years (mean 49 years) and 25 women aged 23–76 years (mean 47 years). Disease extent and activity are shown in Table 1. Seventeen patients were on systemic corticosteroids, 35 on sulphasalazine or another 5-aminosalicylate (5-ASA) derivative, eight on topical steroids, three on azathioprine and seven on no treatment. Activity was graded according to Truelove and Witts [14].

Crohn's disease

Thirty-three patients with Crohn's disease were studied: 14 men aged 20–77 years (mean 50 years) and 19 women aged 17–68 years (mean 48 years). Disease distribution and activity are shown in Table 1. For grading of disease activity, the Crohn's disease activity index (CDAI) was used [15]. Seventeen patients were taking oral corticosteroids, 12 were on sulphasalazine or

Table 1. Clinical characteristics of patients with ulcerative colitis and Crohn's disease.

Clinical characteristics	Ulcerative colitis	Crohn's disease
Disease activity		
Active	19	9
Inactive	30	24
Systemic steroids or immunosuppressive therapy		
Yes	20	18
No	29	15
Disease extent		
Proctitis	13	
Left side	25	
Subtotal	6	
Pancolitis	5	
Disease site		
Small bowel only		13
Ileocolonic		11
Colorectal		9

another 5-ASA derivative, seven on azathioprine, two on 6-mercaptopurine and 10 on no treatment.

Primary sclerosing cholangitis

Four patients with primary sclerosing cholangitis were studied: three men aged 27–64 years and one woman aged 35 years. The diagnosis was made by endoscopic retrograde cholangiography. In two patients cirrhosis of the liver had been previously diagnosed. Two had also suffered from ulcerative colitis, one from Crohn's disease and one from vasculitis.

Vasculitic conditions

Sera from 16 patients with known ANCA positivity on indirect immunofluorescence, with Wegener's granulomatosis, microscopic polyarteritis, crescentic glomerulonephritis and Churg-Strauss syndrome, were included for comparison.

Healthy controls

Thirty-five individuals were included as healthy controls: 17 women aged 24–57 years and 18 men aged 21–56 years.

Preparation of polymorphonuclear leukocytes

Heparinized blood of healthy volunteers was layered onto methyl cellulose hypaque for 15 min. The top layer was diluted 1:3 with phosphate-buffered saline (PBS; pH 7.2) and centrifuged for 5 min at 1000 r.p.m. Red blood cells in the granulocyte-containing pellet were lysed by ice-cold distilled water. Typically, 95% of the isolated PMNLs were neutrophils and 5% were eosinophils. Purified neutrophils were either used to make cytospin preparations for indirect immunofluorescence or to prepare azurophilic granules for ELISA.

Indirect immunofluorescence assay

Cytopreparations were fixed with 96% ethanol at 4°C for 5 min and stored at -20°C until use. Slides

were incubated for 30 min in a moist chamber with 20 μ l serum samples diluted 1:16 with PBS. The slides were then washed with two changes of PBS for 5 min each and incubated for a further 30 min with polyvalent fluorescein-conjugated rabbit antibody to human immunoglobulin (Ig; DAKO, Glostrup, Denmark; diluted 1:40 with PBS).

After another wash, slides were mounted in glycerol and then read by two independent observers. Two normal sera, two positive cANCA and pANCA sera from patients with vasculitis and a control without the addition of sera were included with each batch of sera tested.

Antinuclear antibodies

Antinuclear antibodies were sought by standard techniques using HEP-G2 cells as a substrate, and a reaction with serum dilution of 1:40 or higher was considered positive.

Antigen preparations

PMNLs were disrupted by nitrogen cavitation at 2415 kPa for 20 min at 4°C. After centrifugation, the granulocyte components in the supernatant were separated on a discontinuous Percoll gradient (Sigma, Poole, England, UK). The α -granules were collected and disrupted by adding 1% Triton X-100. The α -antigen preparation was stored at -20°C and used as a substrate in a 1:1000 suspension in carbonate buffer (pH 9.6) for ELISA.

Lyophilized preparations of human PMNL myeloperoxidase (5 μ g/ml; Calbiochem, La Jolla, California, USA), human neutrophil elastase (1 μ g/ml; Calbiochem), human neutrophil cathepsin G (1 μ g/ml; Calbiochem), human neutrophil lysozyme (1 μ g/ml; Sigma), human milk lactoferrin (5 μ g/ml; Sigma) were dissolved in carbonate buffer pH 9.6 and used for ELISA. The purity of the antigens was confirmed by detection of a single band of appropriate molecular weight on sodium dodecylsulphate polyacrylamide gel electrophoresis. In the case of cathepsin G and elastase, which have similar molecular weights (29 and 30 kDa, respectively), Western blotting with anti-elastase antibodies was performed to demonstrate the lack of contamination.

Enzyme-linked immunosorbent assays

ELISA plates (Nunc; Life Technologies, Paisley, Scotland, UK) were coated with α -antigen, myeloperoxidase, elastase, cathepsin G, lactoferrin and lysozyme in suspensions as described above (100 μ l per well) at 4°C overnight. Plates were washed with PBS with 0.05% Tween-20 and later saturated with PBS/Tween/1% bovine serum albumin (BSA) for 1 h at 37°C. Sera were diluted 1:100 in PBS/Tween/1% BSA and applied for 1 h at 37°C (100 μ l per well). Plates were then washed (as above) and incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma; diluted 1:1000) for 1 h at 37°C. After washing, p-nitrophenyl phosphate (Sigma) in carbonate buffer with 5 mmol/l MgCl₂ was added. Absorbance was read at 405 nm when the positive

reference sample had reached an optical density of 1.2–1.5. The results were expressed as a percentage of a positive control serum from a patient with a high level of the relevant antibody. Sera were regarded as positive if the absorbance value was greater than 3 SD above the mean absorbance values for the control group. None of the control samples fell above this cutoff point. All positive sera were retested and confirmed positive in repeat assays. Testing of the reproducibility of this ELISA system revealed an intra-assay variability of approximately 5% and an inter-assay variability of 6–11% [16].

Statistical analysis

Differences in antibody levels for each antigen between the different patient groups was assessed by Student t-test. Student t-test was also used for assessment of differences between subgroups of patients with different clinical features. Linear correlations between the levels of antibodies to different antigens were sought in each group of patients, comparing, for example, anti-elastase antibody levels with anti-lysozyme antibody levels. Ten antigen-pair comparisons were possible. In assessing statistical significance, correction was made for the number of comparisons involved.

Results

Indirect immunofluorescence assay

Positive indirect immunofluorescence on ethanol-fixed granulocytes was found in sera of 20 out of 49 (41%) patients with ulcerative colitis and in only three out of 33 (10%) patients with Crohn's disease. Among the ulcerative colitis patients, four (20% of positive patients) had an unequivocal, classical pANCA pattern and four (20%) were cANCA-positive. The other 12 (60%) were classified as xANCA-positive. Sera from all three positive patients with Crohn's disease were pANCA-positive, and sera from three out of the four patients with PSC were pANCA-positive. All 16 samples from patients with vasculitis were positive, having been selected for their positive ANCA pattern: 10 were pANCA-positive and six were cANCA-positive. All control sera were negative.

Specific antibody levels

We tested sera for reactivity against isolated crude α -antigen, components of α -granules (myeloperoxidase, leukocyte elastase, cathepsin G and lysozyme) and lactoferrin, a component of β -granules. The proportions of patients in whose sera IgG antibodies directed against different neutrophil antigens were detected is shown in Table 2. Antibodies to one or more of the PMNL-associated antigens were detected in 39 out of 49 (69%) patients with ulcerative colitis and 13 out of 33 (39%) patients with Crohn's disease. The low proportion of patients with positive ELISA tests amongst the patients with vasculitis reflects the fact that antibodies to α -granules or to proteinase 3 were not determined.

Table 2. Frequencies of immunoglobulin G antibodies against specific antigens in sera of patients with ulcerative colitis, Crohn's disease, primary sclerosing cholangitis and vasculitis.

	n (%)					
	α -Granules	Myeloperoxidase	Leukocyte elastase	Cathepsin G	Lactoferrin	Lysozyme
Ulcerative colitis (n = 49)	17 (35)	7 (14)	23 (46)	23 (46)	20 (41)	26 (53)
Crohn's disease (n = 33)	4 (12)	1 (3)	6 (18)	3 (12)	1 (3)	13 (39)
Primary sclerosing cholangitis (n = 4)	-	0 (0)	3 (75)	2 (50)	3 (75)	3 (75)
Vasculitis (n = 21)	-	4 (19)	4 (19)	2 (9)	1 (5)	0 (0)

Among patients with ulcerative colitis, levels of IgG antibodies against α -granules, elastase, cathepsin G, lactoferrin and lysozyme were significantly higher ($P < 0.01$) than among controls. Levels of IgG antibodies against myeloperoxidase, however, did not significantly differ from those in the normal group. In Crohn's disease patients, only anti-lysozyme antibody levels were significantly higher than controls ($P < 0.01$).

Table 3. Linear correlations between enzyme-linked immunosorbent assay readings for pairs of specific antibodies (Ab) in ulcerative colitis.

	P*	r
Anti- α -granules versus		
Leukocyte elastase Ab	0.0015	0.87
Cathepsin G Ab	0.0015	0.929
Lactoferrin Ab	0.0015	0.84
Lysozyme Ab	0.0015	0.605
Myeloperoxidase Ab	0.036	0.468
Anti-leukocyte elastase versus		
Cathepsin G Ab	0.0015	0.913
Lactoferrin Ab	0.0015	0.908
Anti-cathepsin G versus		
Lactoferrin Ab	0.0015	0.485
Lysozyme Ab	0.015	0.405
Anti-lactoferrin versus		
Myeloperoxidase Ab	0.02	0.24

*Correlation coefficients were significantly different from zero. Other correlations (anti-leukocyte elastase versus lysozyme Ab, anti-leukocyte elastase versus myeloperoxidase Ab, anti-cathepsin G versus myeloperoxidase Ab, and anti-lactoferrin versus lysozyme Ab) were not significant.

Correlations between titres of different antibodies and IgG levels

Within patient groups, we determined correlations between antibody levels to different pairs of antigens. In ulcerative colitis, there were a number of highly significant correlations (Table 3). As anticipated the levels of antibodies to α -granules correlated positively with those of antibodies to the specific α -granule constituents elastase, cathepsin G, lysozyme and myeloperoxidase, although the correlations with elastase and cathepsin G were more marked ($r = 0.87$ and 0.92 , respectively) than correlations with the other constituents. In addition, levels of anti- α -granule antibodies also strongly correlated ($r = 0.84$) with levels of antibodies to the β -granule constituent lactoferrin. Between pairs of individual enzymes, the strongest correlation was between antibody levels to the two α -granule constituents elastase and cathepsin G ($r = 0.91$). However anti-elastase and anti-lactoferrin levels showed a similarly strong correlation ($r = 0.9$), whereas the correlation of antibodies to cathepsin G with anti-lactoferrin antibodies was less marked ($r = 0.48$).

In Crohn's disease, vasculitis and control groups, significant correlations between pairs of antibodies were not observed. The strong correlations between antibodies to cathepsin G and elastase, and between antibodies to cathepsin G and α -granules, prominently seen in patients with ulcerative colitis, were not found among patients with Crohn's disease.

No significant correlations were found between the total IgG levels and levels of any specific antibody among any of the groups.

Table 4. Associations between patterns of anti-neutrophil cytoplasmic antibodies (ANCA) and positivity in specific enzyme-linked immunosorbent assay (ELISA) tests in patients with ulcerative colitis.

ANCA	No. patients	ELISA antibodies						
		Anti- α -granules	Anti-myeloperoxidase	Anti-leukocyte elastase	Anti-cathepsin G	Anti-lactoferrin	Anti-lysozyme	Negative*
Cytoplasmic	4	2	0	2	2	2	2	0
Perinuclear	4	2	3	3	3	3	3	1
Atypical	12	5	6	6	6	6	6	7
Negative†	29	8	12	12	12	10	15	6

*Level of specific antibodies was not above normal range. †No ANCA was detected.

Association between immunofluorescence ANCA patterns and specific antibody levels

Table 4 shows the relationship between the immunofluorescence pattern and the presence or absence of antibodies to specific antigens detected by ELISA. Unlike the findings in systemic vasculitis, where, in particular, pANCA correlates well with the presence of anti-myeloperoxidase antibodies [3], there was no correlation between indirect immunofluorescence and the presence or absence of circulating ANCAs against specific targets. There were a substantial number of ANCA-positive sera by immunofluorescence in which the panel of ELISA tests for specific antigens did not identify any targets.

Association between ANCAs and clinical features

No differences emerged with respect to the presence or absence of ANCAs on immunofluorescence testing, or with respect to the levels of antibodies to any specific antigen, when patients with ulcerative colitis or Crohn's disease were stratified with respect to disease activity, disease distribution, type of current medical therapy or previous surgery.

Discussion

Understanding the significance of ANCAs in inflammatory diseases is likely to be enhanced by identification of the specific targets. ANCAs have been most extensively studied in vasculitic conditions. The major target in Wegener's granulomatosis is serum proteinase 3 [17] and in microscopic polyarteritis it is myeloperoxidase [3]. Both of these antigens are constituents of the primary or α -granules of granulocytes, but ANCAs to each of these antigens gives a different immunofluorescence staining pattern [3]. Anti-proteinase 3 gives diffuse cytoplasmic staining (cANCA), whereas anti-myeloperoxidase gives a perinuclear (pANCA) distribution because this antigen diffuses from the granules and concentrates around the nucleus [9]. The association between these disease types and ANCA pattern specificity is, however, not absolute: in pANCA-positive microscopic polyarteritis, antibodies to proteinase 3 and to other neutrophil constituents such as elastase also occur [3], and there are 10–50% of pANCA-positive patients in different series in whom the target antigen has not been defined [9,18,19].

In IBD, the existence of granulocyte-specific antibodies has been known since the 1960s [20]. Most authors describe a pANCA pattern in ulcerative colitis, although a pattern distinct from either p- or cANCA, described as x- or atypical ANCA has now been recognized [6]. Using immunofluorescence there is a wide variation in the reported incidence of circulating ANCAs in patients with ulcerative colitis, from 17 to over 80% [5,8,21–24]; however, there is general agreement that the incidence of ANCAs in Crohn's disease is much less frequent, generally one-third to one-quarter of that in

ulcerative colitis. Our comparative results are 10% in Crohn's disease and 41% in ulcerative colitis, and are typical in this regard. Because both the frequency and the pattern of ANCAs detected by immunofluorescence on neutrophil preparations are strikingly altered by minor changes in fixation techniques [22], it seems likely that alternative techniques using purified target antigens, such as the ELISA technique used here, will be more helpful than cell-based assays in elucidating the significance of ANCAs.

This study used specific antigens to investigate the widest panel of putative targets studied so far in IBD, and studied both primary and secondary granule constituents in particular. We did not study proteinase 3, in part because other studies indicate that it is not a target in IBD [5,8], but also because of the difficulties in obtaining pure antigen. Previous studies in vasculitis have taken anti- α -granule antibodies (which we did measure) as a surrogate for anti-proteinase 3, but this is clearly inadequate as the granules contain many different constituents. Also, we did not study antibody to β -glucuronidase [25,26], again because of difficulties in obtaining pure antigen.

We found positive titres of IgG antibodies (in decreasing order of frequencies, between 53 and 35%) to lysozyme, cathepsin G, elastase, lactoferrin and whole α -granules in ulcerative colitis, and also a low frequency (14%) of antibodies to myeloperoxidase. As previously shown by Romas *et al.* [23] (covering the three antigens myeloperoxidase, cathepsin G and elastase), antibodies of more than one specificity were generally found. In Crohn's disease, we found a relatively high (39%) frequency of antibodies to lysozyme, but a fairly low frequency (18% or less) of antibodies to other targets. Thus, our investigations agree with those of Halbwachs-Mecarelli *et al.* [12] in finding a relatively high frequency of antibodies to cathepsin G, but do not find it to be the only or even the most frequent ANCA target in ulcerative colitis. We also confirm the high incidence of anti-lactoferrin antibodies found by Peen *et al.* [13], but found that elastase and lysozyme are also prominent targets.

Why these antibodies are produced is unclear. As in most other studies [5,8,21,27,28] we did not find any relation between clinical activity and the presence or titre of ANCAs either in general or of defined specificity, and therefore find no evidence that they are of primary pathogenetic importance. We also investigated the possibility that these antibodies arise in IBD merely as a result of systemic release of intracellular antigen, but our findings do not support this suggestion. Because the processes effecting release of antigens from primary and secondary granules differ [29], one might anticipate substantial concordance between the presence of antibodies to antigens from one or other granule type, if they arise merely secondarily to antigen release; however, this was not the case. Although antibodies to different constituents of primary granules were often

found together, in some cases there were even stronger correlations between the presence of antibodies to two proteins derived from different granules. Further evidence that the mere release of intracellular antigen is unlikely to explain ANCA development entirely is the low incidence of all types of ANCA in Crohn's disease, in which — at the very least — similar amounts of antigen as in ulcerative colitis are likely to be released from granulocytes; indeed, granulocyte-derived elastase is present in greater amounts in the circulation of patients with Crohn's disease than ulcerative colitis [30], yet the incidence of anti-elastase antibodies is significantly lower in Crohn's disease patients.

A more likely explanation for the higher incidence of ANCAs in ulcerative colitis is that the development of ANCAs reflects genetic susceptibility, which may underlie the tendency to develop ulcerative colitis, as suggested by groups who identified ANCAs both in ulcerative colitis patients and clinically unaffected family members [31]. However, not all agree [32,33], and it may alternatively be that the genetic susceptibility is to develop types or specificities of antibody, and that this is merely linked to and not causative of ulcerative colitis. Yang *et al.* [34] suggested that susceptibility to develop ANCAs was linked to human leukocyte antigen DR2, but this was not confirmed in a different study [35]. One potential explanation, which we are exploring, is that the ANCAs in ulcerative colitis are associated with the known tendency for the IgG subclass IgG1 to predominate in ulcerative colitis, in contrast to IgG2 in Crohn's disease [36], and indeed there is preliminary evidence that IgG ANCAs are predominantly of the IgG1 subclass [37].

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TABLE 1
Fecal Concentration and Daily Fecal Excretion of Lf, PMN-E, MPO, Lys, Hb, and α_1 -AT in UC and CD

	Control	UC (A)	UC (I)	CD (A)	CD (I)
Fecal concentration ($\mu\text{g/g}$)					
Lf	1.6 \pm 0.4	292.8 \pm 35.1	27.6 \pm 5.6	154.4 \pm 28.5	45.3 \pm 11.6
PMN-E	1.0 \pm 0.3	57.5 \pm 13.9	16.3 \pm 7.3	34.0 \pm 7.7	11.4 \pm 2.8
MPO	2.0 \pm 0.6	205.8 \pm 30.5	38.9 \pm 8.5	346.1 \pm 91.3	70.9 \pm 18.3
Lys	1.4 \pm 0.2	48.4 \pm 7.8	10.1 \pm 1.7	17.6 \pm 3.8	11.7 \pm 2.8
Hb	2.5 \pm 0.7	2523.6 \pm 775.9	10.1 \pm 1.9	149.5 \pm 52.0	49.3 \pm 20.7
α_1 -AT	330.7 \pm 38.3	1502.1 \pm 196.3	555.6 \pm 69.2	7094.7 \pm 1842.7	2845.6 \pm 713.0
Fecal excretion (mg/day)					
Lf	0.21 \pm 0.05	50.2 \pm 5.5	5.1 \pm 1.4	34.3 \pm 6.7	6.0 \pm 1.4
PMN-E	0.13 \pm 0.03	13.6 \pm 4.7	2.4 \pm 1.0	7.4 \pm 1.9	1.7 \pm 0.4
MPO	0.28 \pm 0.09	37.4 \pm 6.2	6.9 \pm 1.6	60.3 \pm 13.7	12.5 \pm 4.1
Lys	0.24 \pm 0.05	8.5 \pm 1.3	2.1 \pm 0.5	3.5 \pm 0.6	2.4 \pm 0.6
Hb	0.39 \pm 0.12	808.6 \pm 338.6	1.7 \pm 0.3	19.2 \pm 5.9	4.6 \pm 1.9
α_1 -AT	56.2 \pm 7.8	383.8 \pm 92.5	96.5 \pm 18.1	1102.9 \pm 218.7	325.4 \pm 62.5

UC (A), ulcerative colitis (active phase); UC (I), ulcerative colitis (inactive phase); CD (A), Crohn's disease (active phase); CD (I), Crohn's disease (inactive phase). Values are expressed as means \pm SEM.

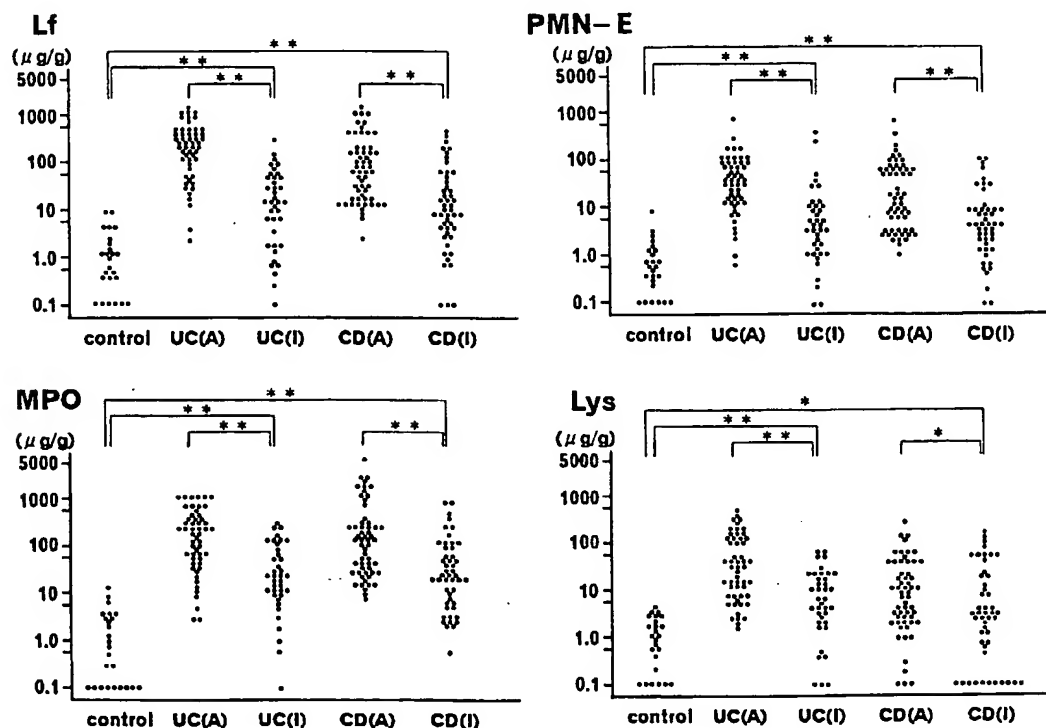


FIG. 3. Fecal concentrations of Lf, PMN-E, MPO, and Lys in UC, CD, and control subjects. UC(A), ulcerative colitis (active phase); UC(I), ulcerative colitis (inactive phase); CD(A), Crohn's disease (active phase); CD(I), Crohn's disease (inactive phase). Student's *t* test was used for statistical analyses: **p* < 0.05, ***p* < 0.01.

tions of neutrophil-derived proteins and the concentrations of Hb and α_1 -AT.

As shown in Figure 4, there were significant correlations on the logarithmic scale between the four neutrophil-derived proteins and Hb. The best correlation ($r = 0.724$) was obtained between fecal Lf and Hb. Fecal Lf, PMN-E, MPO, and Lys concentrations were high in 15, 9, 14, and 14, respectively, of the 25 samples with normal Hb concentra-

tion. Only two samples (inactive UC with internal hemorrhoids) showed no increase in fecal Lf concentration with increases in Hb concentration.

Relationship between the four fecal neutrophil-derived proteins and fecal α_1 -AT in CD

As shown in Figure 5, fecal Lf, PMN-E, and MPO concentrations correlated significantly with α_1 -AT concentra-

UC

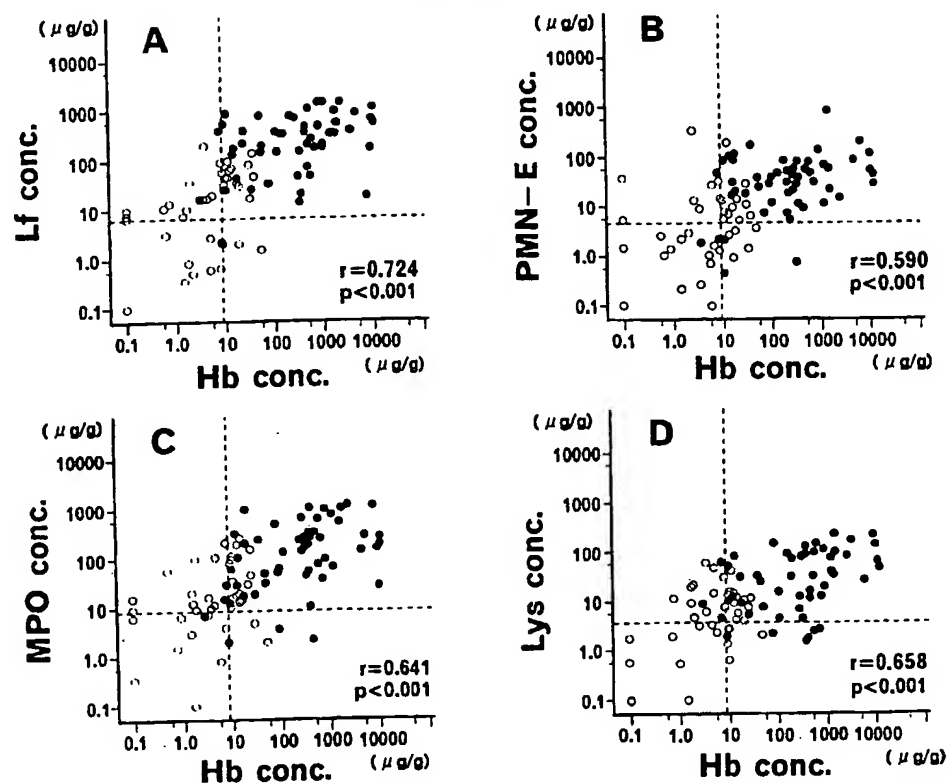


FIG. 4. Correlation between levels of four fecal neutrophil-derived proteins and levels of fecal Hb in UC. A, correlation between fecal Lf and Hb; B, correlation between fecal PMN-E and Hb; C, correlation between fecal MPO and Hb; D, correlation between fecal Lys and Hb. Dotted lines, mean + 2 SD of the control subjects (5.96, 4.37, 8.22, 3.85, and 9.18 $\mu\text{g/g}$ for Lf, PMN-E, MPO, Lys, and Hb, respectively). ●, active phase; ○, inactive phase.

tion on the logarithmic scale, but fecal Lys did not. A good correlation was obtained between fecal Lf or MPO and fecal α_1 -AT. Fecal Lf, PMN-E, and MPO concentrations were high in 19, 10, and 16, respectively, of the 30 samples with normal α_1 -AT concentration. Only six samples and one sample, respectively, showed no increase in fecal Lf and MPO concentrations with increases in α_1 -AT concentration.

DISCUSSION

Evaluation of whether mucosal inflammation is present is very important in the management of patients with IBD (29), because if minimal mucosal inflammation remains in a patient who is evaluated clinically to be in remission, the rate of relapse is high (30, 31). Even colonoscopy and x-ray examination may not detect inflammation if the inflammation is minimal or localized. It is conceivable that feces reflects the state of all sites in the digestive tract, since feces is formed during transit of the bowel.

In evaluating fecal proteins, it is necessary to consider the instability in the intestines or stools to correctly interpret the data. Proteins may be degraded by digestive enzymes and by intestinal bacteria. At low temperatures, fecal proteins are expected to be relatively stable (32). Our *in vitro* study

showed that all four proteins were fairly stable in feces at 4°C. In the present clinical study, therefore, the degradation of fecal protein after defecation was considered to be negligible, because the stool was stored at 4°C immediately after defecation. Generally, for a fecal test to be acceptable in clinical practice, the protein should be stable even at room temperature. We therefore examined the stability at 25°C and 37°C as well. Lf was the most stable and Lys was the most unstable at these temperatures. The carbohydrate content of the Lf molecule may contribute to its good stability.

It is well known that neutrophil granular proteins are released extracellularly when the cells are stimulated (7). Specific granules degranulate in an earlier phase than azurophilic granules after stimulation (33). It is thought that large quantities of neutrophil granular proteins are released extracellularly in the inflamed mucosa of IBD. We carried out an *in vitro* study to investigate which of the four neutrophil granular proteins examined was released extracellularly most efficiently after stimulation. Two different stimuli were used: PMA, an activator of protein kinase C (34), and latex beads, acting as a phagocytic stimulus (35). With either stimulus, Lf was released most efficiently, with Lys being second. Thus, since Lf and Lys are contained in

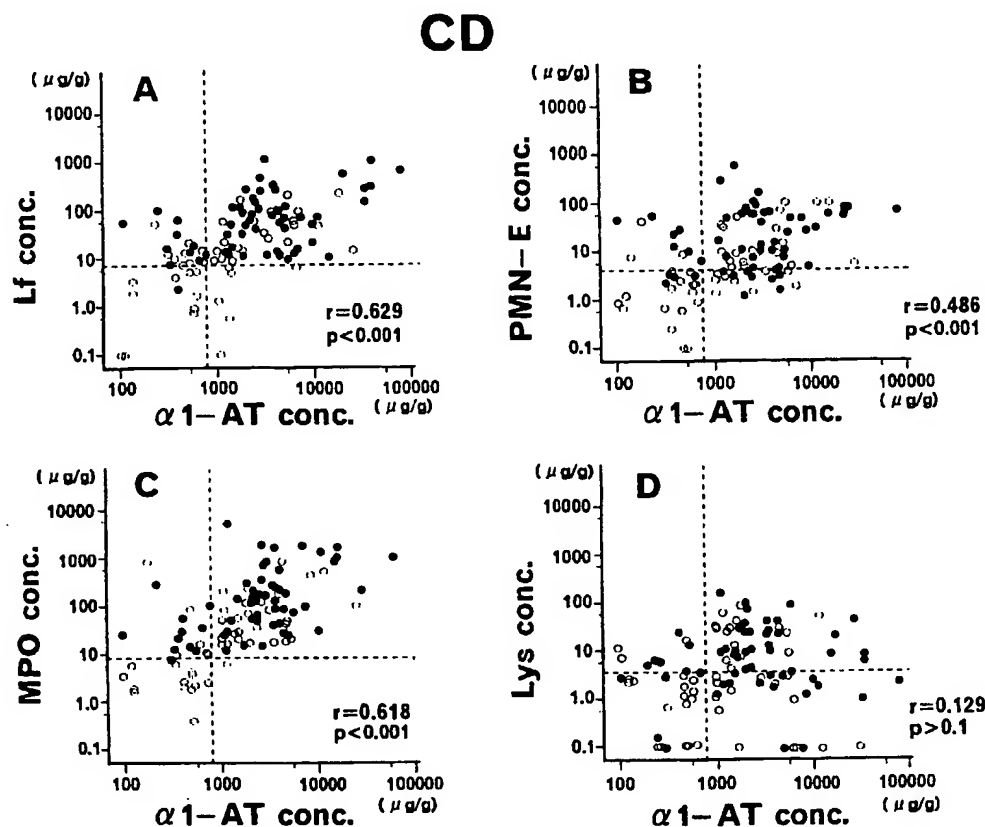


FIG. 5. Correlation between levels of four fecal neutrophil-derived proteins and levels of fecal α_1 -AT in CD. A, correlation between fecal Lf and α_1 -AT; B, correlation between fecal PMN-E and α_1 -AT; C, correlation between fecal MPO and α_1 -AT; D, correlation between fecal Lys and α_1 -AT. Dotted lines, mean + 2 SD of the control subjects (5.96, 4.37, 8.22, 3.85, and 714.0 $\mu\text{g/g}$ for Lf, PMN-E, MPO, Lys, and α_1 -AT, respectively). ●, active phase; ○, inactive phase.

specific granules, it would seem that the specific granules degranulate both more efficiently and earlier than the azurophilic granules. To constitute a superior fecal marker of intestinal inflammation, a protein should be released efficiently from activated neutrophils, as well as being stable in feces. From this point of view, Lf seemed to be the best fecal marker, because it was released from activated neutrophils most efficiently and was the most stable in feces.

The fecal levels of all four neutrophil-derived proteins were significantly greater in active UC and CD than in control subjects. However, the fecal levels of Lys in active CD overlapped, to a great extent, with the values for control subjects. It is most likely that Lys was destroyed to a much greater extent than the other proteins during intestinal transit, since 28 of the 34 CD patients had small intestinal disease. As shown in Table 2, fecal Lys level and levels of the other three fecal neutrophil-derived proteins tested were poorly correlated in CD ($r < 0.5$), whereas good correlations were found between Lf and PMN-E, between Lf and MPO, and between PMN-E and MPO ($r > 0.6$), suggesting that fecal Lys was not a suitable marker for monitoring disease activity in CD.

The fecal levels of all four neutrophil-derived proteins

TABLE 2
Relationship between Four Fecal Neutrophil-derived Proteins in UC and CD

UC		
Lf vs. PMN-E	$p < 0.001$	$r = 0.733$
Lf vs. MPO	$p < 0.001$	$r = 0.883$
Lf vs. Lys	$p < 0.001$	$r = 0.713$
PMN-E vs. MPO	$p < 0.001$	$r = 0.725$
PMN-E vs. Lys	$p < 0.001$	$r = 0.602$
MPO vs. Lys	$p < 0.001$	$r = 0.724$
CD		
Lf vs. PMN-E	$p < 0.001$	$r = 0.665$
Lf vs. MPO	$p < 0.001$	$r = 0.741$
Lf vs. Lys	$p < 0.005$	$r = 0.283$
PMN-E vs. MPO	$p < 0.001$	$r = 0.723$
PMN-E vs. Lys	$p < 0.001$	$r = 0.420$
MPO vs. Lys	$p < 0.001$	$r = 0.454$

Relationship between fecal protein levels were examined on the logarithmic scale. Linear regression analysis was used for correlation analysis.

tested were significantly lower in inactive UC and CD than levels in active disease. However, not a few samples from inactive UC and CD patients showed high fecal levels of the proteins. Inflammation may have been present in the intes-

tinal mucosa in these patients, presumably because, in this study, a patient was defined as having inactive disease when one was not defined as active.

Comparison of UC and CD showed that the levels of fecal neutrophil-derived proteins, except for MPO, were somewhat higher in UC than in CD. This could be explained in terms of the different histological findings in the two diseases; UC is primarily characterized by superficial inflammation with infiltration of neutrophils, and CD by the accumulation of inflammatory cells in deeper layers of the mucosa. In UC, therefore, larger amounts of neutrophil-derived proteins could easily be excreted into the gut lumen. Fecal MPO level in CD was higher than that in UC, MPO was predominant in active CD, and Lf was predominant in active UC. MPO derived from mononuclear cells could contribute to the high fecal MPO level in CD.

We attempted to investigate the relationship between intestinal bleeding and inflammation in UC and the relationship between intestinal protein loss and inflammation in CD. For that purpose, we compared the levels of four fecal neutrophil-derived proteins and Hb in UC, and the levels of the four fecal neutrophil-derived proteins and α_1 -AT in CD. In the relatively large number of patients who showed high fecal neutrophil-derived protein levels but normal Hb and α_1 -AT levels, there may have been a minimal infiltration of neutrophils somewhere in the mucosa. In contrast, in the few patients who showed normal fecal neutrophil-derived protein levels but high Hb and α_1 -AT levels, bleeding and intestinal protein loss may have been present without inflammation. In UC, as shown in Figure 4, fecal Lf showed the best correlation with fecal Hb. High fecal Lf levels but normal Hb levels were found in 15 of 91 stool samples from UC patients. On the other hand, only two stool samples (inactive UC) showed no increase in Lf concentration with increases in Hb concentration. Thus, it appears that, by measuring fecal Lf, one would be least likely to miss a patient with bleeding, and this measurement could also reflect well any minimal intestinal inflammation without bleeding. These findings suggest that fecal Lf would be the most useful marker for monitoring disease activity in UC.

In CD, as shown in Figure 5, fecal MPO and Lf showed good correlations with fecal α_1 -AT. When fecal Lf was used as a marker of inflammation, six patients (all assessed as in the inactive phase) were considered to have minimal inflammation without intestinal protein loss. In contrast, when fecal MPO was used as a marker of inflammation, only one patient showed intestinal protein loss without inflammation.

Our clinical study indicated that, for evaluating the presence of minimal intestinal inflammation, fecal Lf was the most useful marker in UC, and fecal MPO and Lf were the most useful markers in CD. Fecal Lys was not a suitable marker in CD.

In conclusion, both our clinical and *in vitro* studies suggest that Lf is the most suitable neutrophil-derived fecal marker of inflammation for clinical application.

ACKNOWLEDGMENTS

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Fecal Lactoferrin as a Marker for Disease Activity in Inflammatory Bowel Disease: Comparison with Other Neutrophil-derived Proteins

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Objectives: 1) To investigate which neutrophil-derived proteins in feces most accurately reflect disease activity in inflammatory bowel disease. 2) To examine the extracellular release of these proteins by activated neutrophils and their stability in feces by *in vitro* study. **Methods:** We studied 41 patients (91 samples) with ulcerative colitis (UC), 34 patients (105 samples) with Crohn's disease (CD), and 25 control subjects. Fecal levels of lactoferrin (Lf), polymorphonuclear neutrophil elastase (PMN-E), myeloperoxidase (MPO), and lysozyme (Lys) were measured by ELISA. We also measured fecal hemoglobin (Hb) and α_1 -antitrypsin (α_1 -AT), useful markers of disease activity in UC and CD, respectively. For the *in vitro* study, blood samples were stimulated with phorbol myristate acetate or latex beads. For the assessment of stability, homogenized stool samples were stored at 4°C, 25°C, and 37°C for various periods. **Results:** 1) Fecal Lf, PMN-E, MPO, and Lys concentrations were significantly increased in the active phase of the disease compared to the inactive phase in both UC and CD. 2) Fecal Lf, PMN-E, MPO, and Lys concentrations correlated significantly with fecal Hb concentration in UC, whereas fecal Lf, PMN-E, and MPO concentrations correlated significantly with α_1 -AT concentration in CD. In UC, fecal Lf, PMN-E, MPO, and Lys concentrations were high in 15, 9, 14, and 14 samples, respectively, of 25 samples with normal Hb concentration. In CD, fecal Lf, PMN-E, and MPO concentrations were high in 19, 10, and 16 samples, respectively, of 30 samples with normal α_1 -AT concentration. 3) The extracellular release of Lf was the most efficient and this molecule was the most stable in feces. **Conclusions:** Both our clinical and our *in vitro* studies suggested that Lf is the most suitable of these proteins to use as a neutrophil-derived fecal marker of inflammation for clinical application.

INTRODUCTION

For the assessment of disease activity in inflammatory bowel disease (IBD), symptoms, signs, and laboratory ex-

aminations are essential but not sufficient. Barium enema examination, colonoscopy, and histological examination are very useful for such assessment, but these tests create a heavy burden for the patient and may exacerbate the mucosal inflammation. In contrast, fecal tests are safe and can be performed repeatedly. We have recently shown that the measurement of fecal hemoglobin (Hb) is useful for assessing intestinal disease activity in patients with UC, and the measurement of fecal α_1 -antitrypsin (α_1 -AT) is useful in patients with CD (1). Although these fecal proteins reflect bleeding or protein loss from inflamed intestinal mucosa, they do not directly reflect inflammation. Fecal neutrophil-derived proteins have been the subject of a number of reports (2-6); however, it remains to be clarified which neutrophil-derived proteins in feces most accurately reflect the disease activity of IBD. In the present study, we investigated lactoferrin (Lf), polymorphonuclear neutrophil elastase (PMN-E), myeloperoxidase (MPO), and lysozyme (Lys) in feces. Lf is found in specific neutrophil granules, PMN-E and MPO are found in the azurophilic granules, and Lys is found in both types of granules (7-10).

Lf, an iron-binding protein with a molecular weight of approximately 80,000, is found in various secretions, such as breast milk, tears, and saliva, as well as in specific granules of neutrophils (11-14). Lf is present in the intestinal mucus and has an antibacterial effect (15, 16). We have recently developed an immunochemical test for fecal Lf, utilizing an enzyme-linked immunosorbent assay (ELISA), and have shown that fecal Lf levels are high in patients with colorectal diseases (17).

PMN-E is released extracellularly when the cell is activated, causing tissue damage. It is well known that PMN-E is present in plasma as an elastase/ α_1 -AT complex (18). However, the form of PMN-E in feces is not well defined. Our recent evaluation confirmed that most PMN-E was not complexed with α_1 -AT in feces (19).

MPO catalyzes the reaction of hydrogen peroxide and halide ions to produce cytotoxic acids, such as hypochlorous acid (7). These acids play a role in the oxygen-dependent killing of microorganisms and tumor cells. MPO activity in the mucosa has been used as an index of neutrophil infil-

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tration in experimental colitis models (20). However, fecal levels of MPO in humans have not yet been established.

Lys catalyzes the breakdown of the cell walls of some Gram-negative bacteria (7, 21). Lys is contained not only in neutrophils but also in macrophages, Brunner's glands, and Paneth cells (22, 23). Increased fecal Lys levels have been found in both UC and CD (2, 3). However, fecal Lys levels did not correlate with disease activity in patients with small bowel inflammation (24). These findings may reflect the instability of fecal Lys. However, the stability of Lys in feces has not yet been established.

In the present study, using an ELISA, we measured these four neutrophil-derived proteins in the feces of patients with IBD in the active and inactive phases and compared the usefulness of these proteins as markers of intestinal inflammation. In addition, we investigated the relationship between the levels of these four fecal neutrophil-derived proteins and the levels of fecal Hb and α_1 -AT. We also performed an *in vitro* study to examine the extracellular release of Lf, PMN-E, MPO, and Lys by activated human neutrophils, and the stability of these proteins in feces.

MATERIALS AND METHODS

Extracellular release of Lf, PMN-E, MPO, and Lys by activated human neutrophils in vitro

Five hundred microliters of heparinized blood samples from five healthy subjects was incubated at 25°C for 15 min in the presence of 20–320 ng/ml of phorbol myristate acetate (PMA; Wako Junyaku, Osaka, Japan) or latex beads (Sekisui Chemicals, Osaka, Japan) (0.5 μ m in diameter, $4 \times 10^6 - 2.56 \times 10^8$ /ml) that had been coated with rabbit anti-human IgG (Dakopatts, Glostrup, Denmark) (25). Plasma was obtained by centrifugation at 2000 g for 5 min. The concentration of each protein (Lf, PMN-E, MPO, and Lys) in plasma was measured by ELISA, as described below.

To determine the total amount of each protein present in whole blood, Triton X-100 (1% final concentration) was added to the heparinized blood samples, and the concentration of each protein (Lf, PMN-E, MPO, and Lys) was measured.

The percentage of each protein released extracellularly was obtained by the following equation: Percent extracellular release (%) = $\{(a - c)/(b - c)\} \times 100$, where *a* is the concentration (μ g/ml) in plasma after incubation with latex beads or PMA, *b* is the concentration (μ g/ml) in whole blood treated with Triton X-100, and *c* is the concentration (μ g/ml) in plasma without stimulation.

Subjects

Forty-one patients with UC [age 36.1 ± 16.2 yr (mean \pm SD); nine with proctitis, 11 with left-sided colitis, 21 with pancolitis] and 34 patients with CD (age 28.9 ± 13.4 yr, 12 with small intestine type, 16 with small and large intestine type, six with large intestine type) were evaluated. Thirteen

of 41 UC patients and 16 of 34 CD patients were hospitalized two or more times, and each admission was treated as an independent clinical course. UC was defined as being in the active phase if the patients showed clinical symptoms (rectal bleeding, diarrhea) and/or inflamed colonic mucosa (grade 2 or 3) at colonoscopy (26). Disease activity in CD was assessed according to the Crohn's disease activity index (27), in which a score of more than 150 was considered to represent active disease. The control group consisted of 25 subjects (age 42.2 ± 17.3 yr) with no demonstrated abnormality in the upper or lower digestive tract.

Informed consent was obtained from each subject in accordance with the Helsinki Declaration.

Method of stool collection

Patients were instructed to defecate directly into a polystyrene container (diameter 15 cm; depth 12 cm). The stool samples, collected at 4°C over a period of 48–72 h, were homogenized with a small amount of water, and then stored at -80°C until the time of measurement.

Measurement of fecal Lf, PMN-E, MPO, and Lys by ELISA

One hundred microliters of 0.1 M Tris-HCl buffer (pH 8.4), containing 5 μ g/ml anti-human-Lf antibody (Dakopatts), anti-human-PMN-E antibody (Serotec, Oxford, England), anti-human-MPO antibody (Dakopatts), or anti-human-Lys antibody (Dakopatts), were placed into the well of a 96-well microplate and left overnight at 4°C. The solution was then removed, after which the wells were treated with 0.1 M Tris-HCl-buffered saline (pH 8.0), containing 1% bovine serum albumin, to block nonspecific binding sites. The samples, diluted 100- to 10,000-fold with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1% bovine serum albumin and 0.2% sodium azide, were added to each well. After reaction at 37°C for 1 h, the wells were washed with water. The antibodies noted above were labeled with alkaline phosphatase by the periodic acid-Schiff stain method (28). The samples were then reacted with the respective alkaline phosphatase-labeled antibody. After reaction at 37°C for 1 h, the wells were washed with water. The enzyme reaction test was then performed, and color development was measured with a microplate colorimeter (Sanko Junyaku, Tokyo, Japan) at 510/630 nm. The concentration of these proteins was calculated from a standard curve produced with the authentic proteins (Lf from human milk, Sigma, St. Louis, MO; and PMN-E, MPO, and Lys, Athens Research and Technology Inc., Athens, GA). Coefficient of variations in intra-day assay and inter-day assay for these four proteins were less than 10% and 15%, respectively.

Fecal hemoglobin and α_1 -AT levels were measured by ELISA, as described previously (1).

Stability of Lf, PMN-E, MPO, and Lys in feces

To examine the stability of these proteins in feces, homogenized stool samples were stored at 4°C, 25°C and 37°C

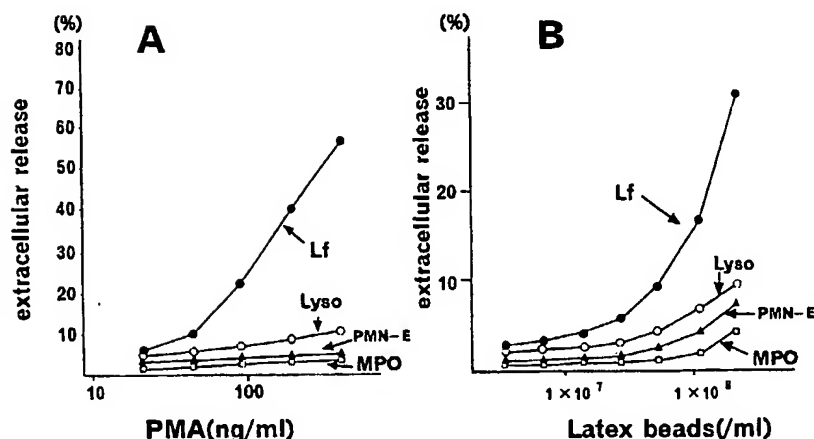


FIG. 1. Extracellular release of Lf, PMN-E, MPO, and Lys from activated human neutrophils *in vitro*. A, neutrophils stimulated with PMA; B, neutrophils stimulated with latex beads.

for 0, 12, 24, 48, 72, and 96 h before freezing and subsequent analysis.

Statistical analysis

Values were expressed as means \pm SEM. Student's *t* test was used to compare the active and inactive phases of both UC and CD. Linear regression analysis was used for correlation analysis. All *p* values were two-tailed; *p* values less than 0.05 were considered statistically significant.

RESULTS

Extracellular release of Lf, PMN-E, MPO, and Lys by activated human neutrophils *in vitro*

As shown in Figure 1, the extracellular release of Lf was the most efficient (Lf > Lys > PMN-E > MPO) when the cells were activated with either PMA or latex beads. After treatment with Triton X-100, the concentration of Lf, PMN-E, MPO, and Lys in whole blood was 6.88 ± 1.05 , 4.55 ± 0.74 , 13.4 ± 2.80 , 3.89 ± 0.86 $\mu\text{g/ml}$, respectively.

Stability of Lf, PMN-E, MPO, and Lys in feces

The concentration of each neutrophil-derived protein was expressed as a percentage of the original concentration at 0 h. The data are shown in Figure 2. All four proteins were stable at 4°C. At 25°C and 37°C, there were differences in stability in the order Lf > PMN-E > MPO > Lys.

Fecal concentration and excretion of the four neutrophil-derived proteins in UC and CD

The values are shown in Table 1 and Figure 3. Significant differences between the active and inactive phases of UC and CD were found in the fecal concentration and daily fecal excretion of all four neutrophil-derived proteins, and the concentration and excretion of the four proteins were significantly greater in inactive UC and CD than in control subjects.

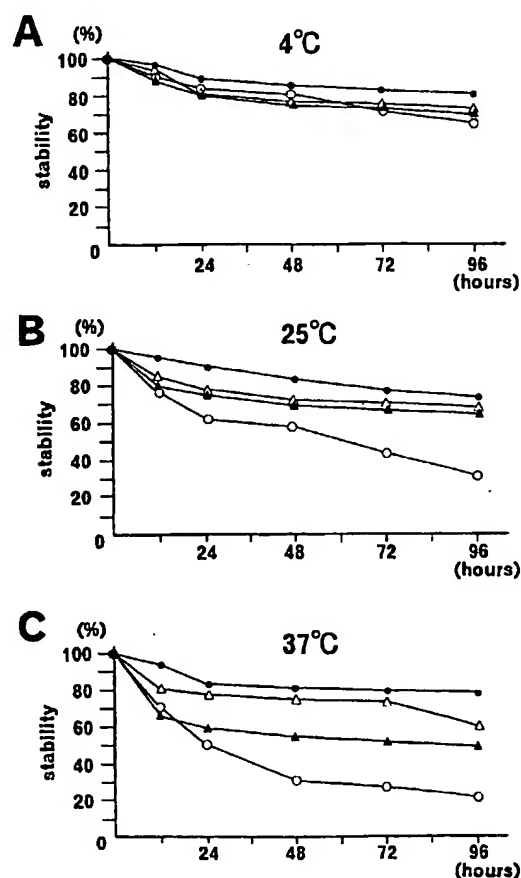


FIG. 2. Stability of Lf, PMN-E, MPO, and Lys in feces: A, at 4°C; B, at 25°C; C, at 37°C. ●, Lf; △, PMN-E; ▲, MPO; ○, Lys.

Relationship between the four fecal neutrophil-derived proteins and fecal Hb in UC

We recently found that fecal Hb and α_1 -AT were useful markers of disease activity in UC and CD, respectively (1). We therefore examined the relationship between concentra-

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Familial and sporadic inflammatory bowel disease: comparison of clinical features and serological markers in a genetically homogeneous population.

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BACKGROUND: The familial occurrence of inflammatory bowel disease (IBD) and the clinical features of familial and sporadic IBD in the genetically homogeneous Finnish population are evaluated. **METHODS:** 257 patients with Crohn disease (CD) and 436 with ulcerative colitis (UC) participated in the study. They were asked whether IBD was present (familial IBD) or absent (sporadic IBD) in their first-degree relatives. Data on the clinical course of the disease were collected from the patient records. Antibodies to *Saccharomyces cerevisiae* (ASCA) and anti-neutrophil cytoplasmic antibodies (ANCA) were determined from serum samples. **RESULTS:** Affected first-degree relatives were found in 15.6% of patients with CD and in 13.8% of patients with UC. In familial cases CD was more often located in the ileum (38% versus 21%) and less often in the ileocolon (35% versus 50%) ($P < 0.05$) than in sporadic cases. A greater percentage of CD patients than UC patients were smokers (47% versus 13%; $P < 0.01$). An elevated level of IgA and/or IgG antibodies for ASCA was found more often in CD patients than in UC patients (59% versus 14%; $P < 0.01$), while pANCA were found more often in UC than in CD patients (48% versus 12%; $P < 0.01$). The combination of pANCA-ASCA+ yielded a sensitivity, specificity and positive predictive value of 48%, 92% and 90%, respectively, for CD, and the combination of pANCA + ASCA- of 55%, 94% and 90%, respectively, for UC. **CONCLUSIONS:** The percentage of familial IBD cases in Finland is comparable to that reported elsewhere in Europe. No important clinical differences between patients with familial and sporadic forms of the disease were found. ASCA is associated with both familial and sporadic CD and pANCA with UC, but low sensitivity diminishes their value as a serological marker of IBD or as a differential diagnostic test between CD and UC.

☐ 1: MLO Med Lab Obs. 2001 Nov;33(11):8-15; quiz 16-9.

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Serologic markers in inflammatory bowel disease (IBD).

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Inflammatory bowel disease (IBD) is a generic term that refers to Crohn's disease and ulcerative colitis. Crohn's disease (CD) is a granulomatous enteritis which can involve the ileum, colon, and other parts of the intestinal tract. The serologic responses seen in Crohn's disease include antibodies to *Saccharomyces cerevisiae*, mycobacteria, bacteroides, listeria and *E. coli*. Many of these organisms may be involved in the pathogenesis of the Crohn's disease. Ulcerative colitis is characterized by inflammation of the mucosa and submucosa of the large intestine. The CD and UC are considered to be distinct forms of IBD; however, there is a subgroup of CD with a UC-like presentation. In recent years, several serologic markers have been found to be useful for the diagnosis and differentiation of CD and UC. These markers include the following antibodies (a) 2pANCA, (b) ASCA, (c) pancreatic antibody, and (d) OmpC antibody. The application of a panel of markers with the use of an algorithm can identify specific subtypes of IBD that have different clinical courses and progression of the diseases. The application of the serologic markers is

Familial and Sporadic Inflammatory Bowel Disease

Comparison of Clinical Features and Serological Markers in a Genetically Homogeneous Population

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Halme L, Turunen U, Heliö T, Paavola P, Walle T, Miettinen A, Järvinen H, Kontula K, Färkkilä M. Familial and sporadic inflammatory bowel disease: comparison of clinical features and serological markers in a genetically homogeneous population. *Scand J Gastroenterol* 2002;37:692–698.

Background: The familial occurrence of inflammatory bowel disease (IBD) and the clinical features of familial and sporadic IBD in the genetically homogeneous Finnish population are evaluated. **Methods:** 257 patients with Crohn disease (CD) and 436 with ulcerative colitis (UC) participated in the study. They were asked whether IBD was present (familial IBD) or absent (sporadic IBD) in their first-degree relatives. Data on the clinical course of the disease were collected from the patient records. Antibodies to *Saccharomyces cerevisiae* (ASCA) and anti-neutrophil cytoplasmic antibodies (ANCA) were determined from serum samples. **Results:** Affected first-degree relatives were found in 15.6% of patients with CD and in 13.8% of patients with UC. In familial cases, CD was more often located in the ileum (38% versus 21%) and less often in the ileocolon (35% versus 50%) ($P < 0.05$) than in sporadic cases. A greater percentage of CD patients than UC patients were smokers (47% versus 13%; $P < 0.01$). An elevated level of IgA and/or IgG antibodies for ASCA was found more often in CD patients than in UC patients (59% versus 14%; $P < 0.01$), while pANCA were found more often in UC than in CD patients (48% versus 12%; $P < 0.01$). The combination of pANCA-ASCA+ yielded a sensitivity, specificity and positive predictive value of 48%, 92% and 90%, respectively, for CD, and the combination of pANCA + ASCA– of 55%, 94% and 90%, respectively, for UC. **Conclusions:** The percentage of familial IBD cases in Finland is comparable to that reported elsewhere in Europe. No important clinical differences between patients with familial and sporadic forms of the disease were found. ASCA is associated with both familial and sporadic CD and pANCA with UC, but low sensitivity diminishes their value as a serological marker of IBD or as a differential diagnostic test between CD and UC.

Key words: Crohn disease; serological markers; ulcerative colitis

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There is increasing evidence for the presence of one or more underlying genes predisposing to the development of inflammatory bowel disease (IBD). In genome-wide scanning studies, several susceptibility loci have recently been identified, especially one present in chromosome 16 and linked to Crohn disease (CD); another is present in chromosome 12 and linked to ulcerative colitis (UC) (1–3). Very recently, the CD-linked chromosome 16 locus was tentatively identified as the NOD2 gene, which is expressed monocytes and is thought to function as an intracellular receptor for microbial components (4–6). In our preliminary genetic study of the Finnish population, we obtained evidence of linkage of IBD to chromosome 3p21 (7). This finding is in accordance with earlier reports in which this particular chromosome 3 region was suggested as a susceptibility locus for IBD (2, 8).

It is not clear whether the process of molecular pathogenesis differs in familiarly occurring cases of IBD ('familial IBD') and in the majority of patients with no evidence of familial clustering ('sporadic IBD'). Phenotypic classification is often confused by great variations of environmental factors. Previous studies have shown that smokers are at a higher risk of developing CD than non-smokers (9). Persistent tobacco smoking is a risk factor for relapsing CD, while treatment with transdermal nicotine seems to have some effect on the clinical manifestations of active UC (10, 11). On the other hand, patients with CD who stop smoking for more than 1 year have been found to have a more benign disease course than if they had never smoked (11). High serum levels of perinuclear antineutrophil cytoplasmic antibodies (pANCA) are strongly correlated with occurrence of UC (12–14), while serum reactivity to cell wall mannan polysaccharide of

Saccharomyces cerevisiae (ASCA) is found to be a serological marker of patients with CD (14–17).

The present study evaluates the occurrence of IBD among the first-degree relatives of patients with histologically proven UC and CD and compares clinical features and postulated risk factors of familial and sporadic forms of IBD originating in the genetically homogeneous ethnic Finnish population with similar living conditions and principles of treatment. Moreover, the role of ASCA and pANCA as diagnostic tools is studied.

Patients and Methods

Consecutive IBD patients treated at the gastroenterological and surgical units of the Helsinki University Hospital and Maria Hospital (a municipal hospital within the Helsinki area) between 1995 and 1998 or consecutive patients with UC who underwent colectomy in the 1990s at the Helsinki University hospital were asked to participate in this study. The diagnosis of CD and UC was established according to standard endoscopic and histological criteria (18). Patients with indeterminate colitis and those with proctitis only were excluded. A questionnaire on the history and details of their disease and the number of their affected and non-affected relatives was given to 1000 patients; 82% returned the questionnaire and consented to participate in the study. Patients reporting parents originating outside Finland or affected relatives other than first-degree relatives were excluded. In families with two or more affected members, the first member returning the questionnaire was termed the proband. The final material comprised 693 IBD patients, 436 suffering from UC and 257 suffering from CD.

Details on the course of the disease in patients and their affected relatives were collected from the patient records. The location of IBD was defined to represent the largest macroscopical and microscopical extent registered at some time during the history of the disease. To classify the severity of the disease, the need for medication and excisional surgery was taken into account. Location of the UC was considered to be left-sided if the colitis was restricted to the sigmoid or descending colon and extensive if either the right or transverse colon was involved. CD was graded using the Vienna Classification (19).

Antibody assays

Antibodies to ASCA and ANCA were examined from serum samples taken from a randomly chosen 100 CD and 100 UC patients and from healthy blood donors (45 for determination of ASCA and 20 for ANCA). The samples were taken during a routine visit at return of the questionnaire, aliquoted, and stored at -20°C until studied. Thus, most of the patients had quiescent disease at the time of serological studies. ASCA of IgA and IgG class were studied by the ELISA technique (Quanta Lite ASCA IgA and IgG kits, INOVA Diagnostics Inc, San Diego, Calif., USA), as

instructed by the manufacturer. The cut-off value for IgA antibodies was 20 units (U) and for IgG antibodies 40 U. Anti-neutrophil cytoplasmic antibodies were studied by the indirect immunofluorescence technique using ANCA Ethanol-fixed Neutrophil Slides (The Binding Site Ltd, Birmingham, UK) as substrates. The sera were diluted 1:10 in phosphate buffered saline, pH 7.2, and the positive samples were titrated to the end-point by 5-fold dilution steps. The samples positive for ANCA were further screened for anti-nuclear antibodies (ANA) at dilution 1:80 using HEp-2 ANA Slides (INOVA Diagnostics Inc.). The positive samples were titrated further by 4-fold dilution steps. Goat anti-human IgG F(ab')₂ (Sanofi Diagnostics Pasteur, Inc., Chaska, MN) or rabbit anti-human IgG, -IgA, -IgM, -kappa, -lambda; (DAKO A/S, Glostrup, Denmark) antibodies coupled with fluorescein isothiocyanate (FITC) were used as secondary antibodies for ANCA or ANA assays, respectively. For immunofluorescence microscopy, an Olympus AX60 microscope equipped with epifluorescence and interference filters for FITC was used. The slides were read by two microscopists (A.M. and T.W.) independently without knowing the origin of the samples. The ANCA antibodies were identified as cytoplasmic (cANCA) or perinuclear (pANCA) using the criteria suggested by the International Consensus Statement on testing and reporting ANCA (17).

Ethics

The study was approved by the ethics Review Committee of Helsinki University Hospital. All patients gave their informed consent.

Statistics

The Pearson chi-squared test and Fisher exact test were used to compare frequencies in groups of patients. Student's *t*-test for unpaired samples was used to compare continuous data between groups of patients. Sensitivity was defined as the probability of a positive test result in a patient with the disease under investigation, and specificity as the probability of a negative result in a patient without the disease. The predictive value of a test result is the probability of a true test in a patient with a given test result.

Results

Of the 257 patients with CD, 40 (15.6%) had familial disease, i.e. at least one first-degree relative suffering from IBD, while 60 (13.8%) of the 436 patients with UC had familial disease. The distributions of the type of IBD in the affected first-degree family members of patients with CD and UC are shown in Table I. Thirty percent of CD probands had one or more first-degree relatives suffering from UC or indeterminate colitis, while 70% of the families were affected purely with CD. In 20 (61%) CD families, the proband's affected members were sibs, while in the remaining 13 (39%) CD families they were child-parent pairs. In three (9%) CD

Table I. Distribution of IBD in affected first-degree family members of patients with familial Crohn disease or ulcerative colitis

Diagnosis of the proband	Type of IBD in the relatives		
	Only CD	Only UC	CD and UC or indeterminated
CD (<i>n</i> = 33)	23	7	3
UC (<i>n</i> = 55)	6	45	4

families there were more than two affected first-degree members—all sibs. Eighteen percent of the UC probands had first-degree relatives suffering from CD or indeterminate colitis, while the rest (81%) had pure UC families. The proband's affected family members were siblings in 30 (55%) UC families, child-parents in 22 (40%) and both in 3 (5%) families. There were five families with more than two affected members, including two families with three or more affected sibs and three families with one affected parent and two or more affected children.

Clinical characteristics of the patients with CD are summarized in Tables II and III. Patients with familial CD at diagnosis were slightly older than patients with sporadic CD (N.S.), and compared to sporadic cases the familial cases more often had involvement restricted to the ileum and less often in both the ileum and colon ($P < 0.05$) (Table II). The frequency of bowel surgery needed was the same between these two CD groups, but a greater number of patients with

Table II. Vienna classification of patients with Crohn disease

Variable	Sporadic (<i>n</i> = 217)	Familial (<i>n</i> = 40)	<i>P</i>
Age at onset			
<40 years	179 (82%)	30 (75%)	N.S.
≥40 years	38 (18%)	10 (25%)	
Location			
Ileum	45 (21%)	15 (38%)	<0.05
Colon	51 (24%)	10 (25%)	
Ileocolon	109 (50%)	14 (35%)	
Upper gastrointestinal tract	12 (6%)	1 (3%)	
Behaviour			
Inflammatory	84 (39%)	14 (35%)	N.S.
Strictureing	78 (36%)	19 (47%)	
Penetrating	55 (25%)	7 (18%)	

sporadic disease had needed medical therapies at least once in the course of the disease (Table II). In contrast, no such difference in requirement of medical therapies was seen between patients with sporadic and familial UC (Table IV).

Of the 257 CD patients, 119 (46%) were smokers and 53 (21%) were ex-smokers or casual smokers, whereas 56 (13%) of the 436 UC patients were smokers and 166 (38%) were ex-smokers or casual smokers ($P < 0.01$). Smoking habits did not show any significant differences in sporadic and familial forms of the two diseases (Table III and IV).

IgA types of ASCA were elevated in 44% of CD patients (median 34, range 5–196) and in 10% of UC patients (median 9, range 3–67), but in none of the controls (median 6, range 3–

Table III. Characteristics of patients with sporadic and familial forms of Crohn disease

Variable	Sporadic (<i>n</i> = 217)	Familial (<i>n</i> = 40)	<i>P</i>
Men (%)	92 (42%)	16 (40%)	N.S.
Median age at onset (years)	26.8 (9–63)	29.2 (16–62)	N.S.
Median duration of IBD (years)	6.8 (0–46)	8.4 (0–24)	N.S.
Extraintestinal	54 (25%)	8 (20%)	N.S.
PSC	4	0	N.S.
Arthritis	33	5	N.S.
Skin	10	3	N.S.
Iritis	23	1	<0.05
Medication			
5-ASA	211 (97%)	36 (90%)	N.S.
Metronidazole	154 (71%)	21 (53%)	<0.05
Steroids	173 (80%)	26 (65%)	N.S.
Immunosuppressives	101 (47%)	15 (38%)	N.S.
Bowel surgery			
Resection	114 (52%)	19 (48%)	N.S.
Once	76	12	
≥Twice	38	7	
Permanent enterostomy	9 (4%)	2 (5%)	N.S.
Indication for surgery			
Emergency	5 (4%)	0	
Complication	99 (87%)	18 (95%)	N.S.
Chronic active disease	9 (8%)	1 (5%)	
Dysplasia/carcinoma	1 (1%)	0	
Perianal fistulae	40 (18%)	6 (15%)	N.S.
Fistulotomy	22	4	
Smoking			
No	75 (35%)	10 (25%)	N.S.
Ex or casual	39 (18%)	14 (35%)	
Yes	103 (47%)	16 (40%)	

Table IV. Characteristics of patients with sporadic and familial forms of ulcerative colitis

	Sporadic (n = 376)	Familial (n = 60)	P
Men (%)	208 (55%)	30 (50%)	N.S.
Median age at onset (years)	28.3 (9–74)	30.6 (11–62)	N.S.
Median duration (years)	8.7 (0–51)	13.2 (0–34)	N.S.
Extraintestinal	92 (24%)	11 (18%)	N.S.
PSC	42	5	N.S.
Arthritis	29	5	N.S.
Skin	2	3	<0.01
Iritis	15	1	N.S.
Location			
Left-sided	130 (35%)	24 (40%)	N.S.
Extensive	246 (65%)	36 (60%)	
Medication			
5-ASA	376 (100%)	56 (93%)	N.S.
Steroids	292 (78%)	49 (82%)	N.S.
Immunosuppressives	26 (7%)	6 (10%)	N.S.
Bowel surgery	123 (33%)	23 (38%)	N.S.
IAA	99	19	
Proctocolectomy	16	2	
Other	8	2	
Indication for surgery			
Emergency	17	6	
Chronic active disease	82	12	N.S.
Dysplasia/carcinoma	24	2	
Smoking			
No	181 (48%)	33 (55%)	
Ex or casual	143 (38%)	23 (38%)	N.S.
Yes	52 (14%)	4 (7%)	

19) ($P < 0.01$) (Fig. 1). IgG types of ASCA were elevated in 46% of CD patients (median 17, range 3–214), in 7% of UC patients (median 8, range 2–124) and in 2% of controls (median 6, range 3–119) ($P < 0.01$) (Fig. 1). Considering both

types of ASCA together, IgA and/or IgG antibodies were elevated in 59% of CD patients (50% of familial and 63% of sporadic, N.S.), 14% of UC patients (11% of familial and 16% of sporadic, N.S.) and 2% of the controls ($P < 0.01$) (Table

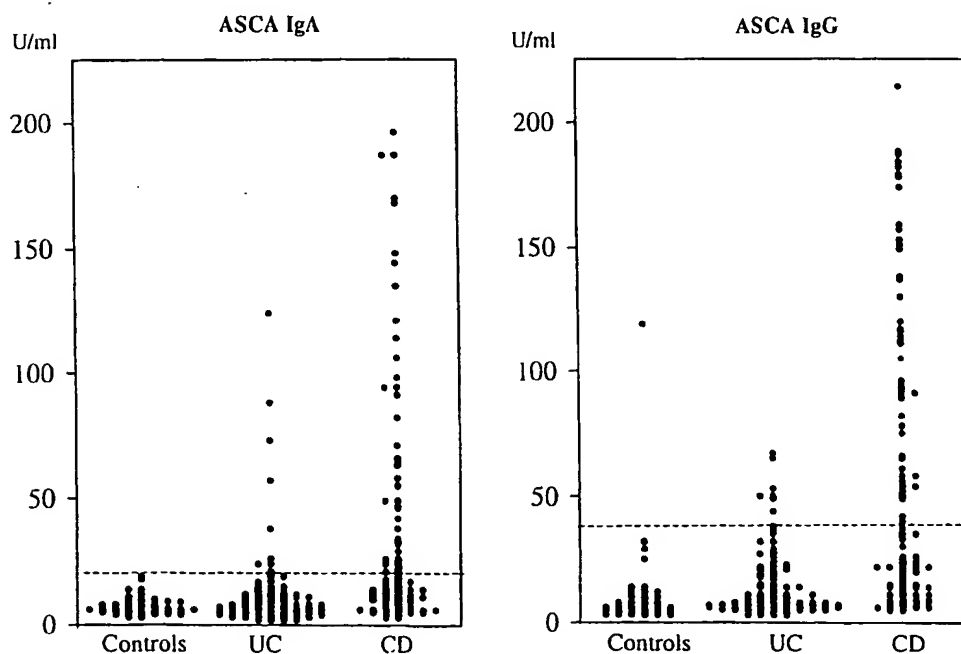


Fig. 1. Titres of IgA and IgG antibodies of ASCA in patients with CD ($n = 100$) and UC ($n = 100$) and healthy controls ($n = 45$).

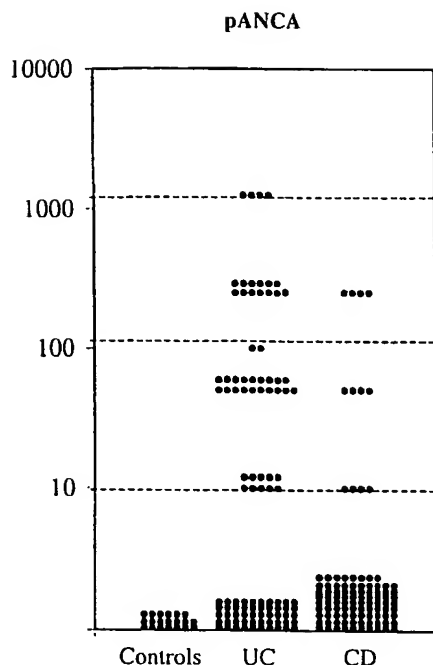


Fig. 2. Titres of pANCA in patients with CD ($n = 100$) and UC ($n = 100$) and healthy controls ($n = 20$).

V). pANCA levels were elevated in 3 familial CD (11%) patients, in 9 (13%) sporadic CD patients, in 19 (51%) familial UC patients, in 29 (46%) sporadic UC patients and were absent in all controls (Table V, Fig. 2). Anti-nuclear antibodies were positive (titre ≥ 80) in 11 (23%) of the pANCA positive UC patients and in 5 (42%) of the pANCA positive CD patients. cANCA level was elevated only in one

sporadic UC patient. ASCA was elevated more often in CD patients than in UC patients ($P < 0.01$) and ANCA more often elevated in UC patients ($P < 0.01$) than in CD patients (Table V). Accuracy data for differentiating CD and UC from controls are given in Table VI.

Discussion

The present study constitutes the first report of clinical features of patients with CD and UC in the Finnish population. In this material, which comprises mainly consecutive patients from a university hospital and in which patients with proctitis only were excluded, we found affected first-degree relatives in 15.6% of patients with CD and in 13.8% of patients with UC. These percentages are similar to the figures reported from other European countries (21–23). Many studies of familial IBD have shown a high degree of concordance for CD or UC within families. In one study, 89% of first-degree relatives of patients with CD also had CD (24), while another series of 240 relative pairs with IBD revealed a concordance rate of 77% for the subtype of the disease (25). In the present series, 81% of affected first-degree relatives with UC also had UC, but only 70% of the probands with CD had affected first-degree relatives with CD. Thus, of 88 IBD families investigated 45 (51%) were UC and 23 (26%) CD families, while the remaining 20 (23%) families were considered as mixed families.

Familially occurring CD has been reported to be associated with a lower proportion of patients with an exclusive colonic involvement, with an increased proportion of patients with small-bowel involvement (26–28) and with an increased frequency of abscess or perforation (23). In the present series,

Table V. Prevalence of elevated ASCA and pANCA titres in patients with familial and sporadic forms of CD and UC

	<i>n</i>	ASCA	pANCA	ASCA and pANCA
Crohn disease				
Familial	28	14 (50 %)	3 (11 %)	0
Sporadic	72	45 (63 %)	9 (13 %)	4 (6 %)
Total	100	59 (59 %)*	12 (12 %)*	4 (4 %)
Ulcerative colitis				
Familial	37	4 (11 %)	19 (51 %)	2 (5 %)
Sporadic	64	10 (16 %)	29 (46 %)	6 (9 %)
Total	100	14 (14 %)*	48 (48 %)*	8 (8 %)

* $P < 0.01$.

Table VI. ASCA and pANCA results for diagnosing either UC or CD in patients with IBD

Test	UC (<i>n</i> = 100)	CD (<i>n</i> = 100)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
pANCA ¹	48	12	48	88	80	63
ASCA ²	14	59	59	86	81	68
pANCA+ASCA ¹	48	8	48	92	86	64
pANCA-ASCA ²	6	55	55	94	90	68

¹ For UC; ² for CD.

a familial type of CD was most often located in the ileum, while in patients with sporadic CD the most common location was the ileocolon. A minor percentage of patients with familial CD had needed medical treatment, but the frequency and indications of bowel surgery were the same. This is in accordance with a recent report suggesting that a positive family history of CD does not increase the severity of CD in affected patients (23).

The clinical course of UC was similar in patients with sporadic and familial types of disease (Table IV). In both types, about two-thirds of the patients had extensive colitis, with no differences in the drug requirements, and in about one-third of patients a colectomy had been performed. Another series of familial UC patients reported an increased prevalence of total colitis (29), whereas another study disclosed no difference in the extent of the colitis (30). Genetic influences have been suggested to be of greater importance in modifying the course of CD than that of UC (31, 32), and in fact there are very few reports on the features of familial versus sporadic types of UC.

A greater percentage of probands with UC were non-smokers or ex-smokers in comparison to probands with CD (87% versus 54%), which is in accordance with previous reports (9–11). No difference in the smoking history was found between the familial and sporadic forms of UC or CD.

The reported prevalence of elevated IgA or IgG antibodies for ASCA varies from 52% to 68% in patients with CD and from 0% to 12% in patients UC (15–17, 33). In the present series the corresponding figures were 59% for patients with CD and 14% for patients with UC, and more than half of the latter patients were also positive for ANCA. The prevalence of ASCA tended to be lower in familial than in sporadic CD and UC groups, which does not support the assumption that ASCA is marker of a genetic rather than environmental component underlying IBD. In the present series of patients the prevalences of pANCA, 48% in UC patients and 12% in CD patients, were in accordance with other reported series (12–15, 34). The fact that serum samples were taken during a routine visit when the disease activity was low seemed not to have an influence on the results. This is in accordance with another report in which no relation was found between the titre of pANCA in UC (or CD) patients and any of the following: disease activity, duration of illness, localization, extent of disease, previous bowel operations or medical treatment (34). Our data indicate that also in Finnish patients ASCA is associated with CD and pANCA with UC similar to reports from other European populations (15, 34, 35), but the value of a combination of these tests in the differential diagnosis of IBD is limited due to low sensitivity rates.

In conclusion, the occurrence (approximately 13%) of UC and CD in the first-degree relatives of Finnish IBD patients is comparable to the estimates from other studied European populations. Clinical differences between patients with familial and sporadic forms of IBD were minor. ANCA and

ASCA titres among familial and sporadic UC and CD patients were similar.

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Role of Serology and Routine Laboratory Tests in Childhood Inflammatory Bowel Disease

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Summary: *Introduction:* Serology is reported to be helpful in evaluating children for inflammatory bowel disease (IBD), and distinguishing chronic ulcerative colitis (CUC) from Crohn's disease (CD). The markers include perinuclear staining antineutrophil cytoplasmic antibody (pANCA) for CUC and anti-*Saccharomyces cerevisiae* antibody (ASCA) for CD. In the clinical setting, hemoglobin (Hgb) and erythrocyte sedimentation rate (ESR) are commonly performed for screening symptomatic children for IBD. We examined whether there was an additional benefit of serology in addition to specific symptoms and routine laboratory tests in screening for IBD. *Method:* Medical record data was reviewed on children investigated for IBD from February 1999 to April 2001. Children were included if they had blood analyzed for pANCA and ASCA, Hgb, ESR, and colonoscopy as part of their assessment. *Results:* Of 177 cases reviewed, 51 were diagnosed with CUC, 39 with CD, and 26 other inflammatory conditions. Visible rectal bleeding was the most discriminating symptom (occurred in 60/90 cases of IBD and 5/61 without IBD). There was a significant difference between the proportion with CUC positive for pANCA (42/51) and those with abnormal Hgb and ESR (30/51) ($p < 0.05$), but

not between children with CD who were ASCA positive (18/39) and those with abnormal Hgb and ESR (26/39) ($p = 0.27$). The sensitivity and specificity of combined pANCA and ASCA was 68% and 92%, respectively. For the combination of Hgb, ESR, and the presence of rectal bleeding the respective values were 86% and 67%. Serology combined with Hgb and ESR and rectal bleeding as independent factors significantly ($p < 0.05$) improved sensitivity (89%) but reduced specificity (60%). Screening with the combination of rectal bleeding, Hgb, and ESR identified 86% (77/90) patients with IBD prior to an endoscopic procedure. A further 3 of 90 (3.3%) screened positive with the addition of serology. *Conclusion:* Serology tests have a high degree of specificity for IBD while routine laboratory test have a higher sensitivity. When serology is combined with rectal bleeding, Hgb, and ESR, the sensitivity of screening children for IBD is significantly improved. However the large majority of children with IBD can be identified with a clinical history and routine laboratory tests as needing an endoscopic procedure with little benefit of adding serology. **Key Words:** Serology—Inflammatory bowel disease—Screening.

INTRODUCTION

Readily available laboratory tests are used to screen children for inflammatory bowel disease (IBD) before an endoscopic procedure (1). A positive screen includes a decrease in hemoglobin (Hgb) or abnormal red blood cell indices as a result of inflammation, nutrient deficiency, or blood loss, and increased erythrocyte sedimentation

rate (ESR) from inflammation. These tests do not distinguish between chronic ulcerative colitis (CUC) and Crohn's disease (CD). The most widely reported serological marker for diagnostic testing in IBD is antineutrophil cytoplasmic antibody (ANCA), which occurs in 80% of individuals with CUC (2,3). The characteristic ANCA associated with CUC has a diffuse cytoplasmic pattern with perinuclear highlighting (pANCA) under indirect immunofluorescent microscopy of neutrophils, which disappears after deoxyribonuclease (DNase) treatment (4). Antibodies that recognize mannose sequences in the cell wall of the yeast *Saccharomyces cerevisiae*

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(ASCA) have been reported in 50% to 80% of sera from patients with CD depending on the yeast strain used (3,5). Combined pANCA and ASCA has a high degree of sensitivity and specificity for differentiating between CUC and CD in adults with IBD (5). In a study using serum from children with IBD, the combination of pANCA and ASCA had a sensitivity of 71% in the diagnosis of IBD (6).

We hypothesized that the available serology does not increase the yield of preendoscopic screening beyond that of routine laboratory tests and clinical findings. To test this hypothesis, we identified children who had been screened with serology and compared results of serology with routine laboratory tests and the most discriminating symptoms. We then combined the results to examine whether serology increased the number of children screened positive beyond routine laboratory tests and symptoms in selecting children for an endoscopic procedure. Sensitivities, specificities, and predictive value of serology were similarly compared with routine laboratory and clinical parameters and again combined to see if there was a significant improvement on existing parameters for screening symptomatic children.

MATERIALS AND METHODS

Data was analyzed retrospectively on children who had been evaluated for IBD between February 1999 and April 2001 by one of eight pediatric gastroenterologists at either the University of Minnesota in Minneapolis or Children's Hospitals of Minneapolis and St. Paul. Patient charts were reviewed between 6 and 30 months after initial investigation. Chart review included symptoms at presentation, investigations performed, and established diagnosis (Tables 1, 2). The study was approved by the Institutional Review Boards of the Fairview University Medical Center and Children's Health Care of Minneapolis and St. Paul.

Patient Selection

The study was limited to 177 children who had both pANCA and ASCA performed (the denominator for the

TABLE 1. Demographics and diagnoses for children evaluated for inflammatory bowel disease

	Chronic ulcerative colitis	Crohn's disease	No inflammatory bowel disease ^a
N	51	39	61
Sex M/F	24/27	21/18	34/27
Age range	3-18	7-18	3-18
Mean age	12.8	13.2	12.1

^a No documented evidence of inflammatory bowel disease at the time of chart review.

study) through Prometheus Laboratories. The only additional criteria for patient selection were an automated complete blood count (CBC), ESR, and a colonoscopy with biopsies during the initial clinical assessment. All pediatric gastroenterologists in the city were included. Cases were not necessarily in sequence. Diagnosis was assigned by the gastroenterologist involved in the patient's care from clinical history, investigative and endoscopic findings, and histopathology. Typically serology was performed during the initial investigations for IBD, and physicians were not blind to results of serology. The group with IBD represented 92% of all new diagnoses in the pediatric population at the University of Minnesota, suggesting that serology was widely used for diagnostic purposes. Cases were not necessarily in sequence. The study population represented 89% of serology performed through Prometheus. The remaining 11% had incomplete data sets according to the above criteria (mainly lack of colonoscopy), suggesting that the physician was less convinced about the possibility of IBD in this group. Assuming that these individuals did not have IBD, the results were likely to improve specificity of serology and routine laboratory tests if they were included. Children who were investigated for IBD and did not have serology performed were not included. An analysis of all children diagnosed with IBD would have helped to determine the selectivity of patients undergoing serological testing; however, it would not have helped with the aim of the study, i.e., a comparison of the laboratory tests. Biopsies of the terminal ileum had been obtained in all individuals without a diagnosis of IBD at follow-up (group labeled "no IBD" in Tables 1 and 2). Five cases were included where severe inflammation limited the colonoscopy to the splenic flexure, with a clear diagnosis on histology, and four of five patients had CUC.

Laboratory Testing

A positive screen for IBD included an Hgb (STKS, Coulter, FL, U.S.A. [used at the University of Minnesota] and Cell-Dyn, Abbott Laboratories, CA, U.S.A. [used at Children's Healthcare]) below the cut-off for age and/or Westergren ESR (Mini-Ves, Vaga Biomed, RI, U.S.A.) elevated above normal for age. The normal lower limit for Hgb was 10.5 g/dl for 1 to 10 years of age and 11.5 g/dl if older than 10 years. The ESR upper limit of normal was 15 mm/hr for 0 to 16 years of age and males older than 16 years, and 20 mm/hr females older than 16 years.

The Prometheus Laboratories (San Diego, CA, U.S.A.) method for pANCA and ASCA has been described previously (6). Results were considered positive as designated by Prometheus Laboratories. Briefly, their

TABLE 2. The number of children with specific symptoms, positive routine laboratory tests, and positive serology

	Chronic ulcerative colitis	Crohn's disease	No inflammatory bowel disease	p value ^a
N	51	39	61	
Main symptoms				
Diarrhea	37	21	18	<0.05
Visible rectal bleeding	37	10	5	<0.05
Pain	34	27	46	0.35
Weight loss	5	13	5	0.05
Laboratory results				
Low Hgb	21	16	6	<0.05
Elevated ESR	21	23	12	<0.05
Abnormal Hgb or ESR	30	26	15	<0.05
Rectal bleeding and/or abnormal Hgb/ESR	47	30	20	<0.05
Serology				
pANCA+	42	2	3	<0.05
ASCA+	2	18	2	<0.05
Combined				
Serology and/or abnormal Hgb/ESR	46	29	18	<0.05
Serology and/or abnormal Hgb/ESR or rectal bleeding	49	31	23	<0.05

^a Comparison of children with chronic ulcerative colitis and Crohn's disease (combined) with those without inflammatory bowel disease using the chi-squared test.

Hgb, hemoglobin; ESR, erythrocyte sedimentation rate; pANCA, perinuclear antineutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody.

ASCA (CD-Dx-1) based on well-defined positive and negative controls, is an enzyme-linked immunosorbent assay (ELISA). Results are positive if immunoglobulin (Ig)A antibodies are above 20 EU/mL and or IgG antibodies are above 40 EU/mL. Immunoglobulin IgA levels to rule out the possibility of IgA deficiency as cause of false negative ASCA were not obtained as part of the clinical assessment. The pANCA (UC-Dx-1) initial ELISA is followed by immunofluorescence for perinuclear staining in samples above the laboratory cut-off. The patients were not further categorized on ELISA titer levels or DNase immunofluorescence as part of our investigation.

Statistical Analysis

Children in whom CUC and CD were diagnosed were compared with children who did not have IBD at the

time of chart review (Tables 1–3). Sensitivity was defined as the percentage of true positives, and specificity as the percentage of true negatives. The predictive value of a positive test was defined as the percentage of individuals with a positive test who had disease and the predictive value of a negative test as the percentage of patients with a negative tests in the absence of disease (5). The chi-squared test was used to compare symptoms, laboratory and serology results, and their combinations (Minitab; Addison Wesley Longman, MA, U.S.A.).

Data from 26/177 children diagnosed with indeterminate colitis (n = 15), eosinophilic colitis (n = 7), and infectious colitis (n = 4) were not included in the statistical analysis. For the latter two disorders, the results would be expected to be dichotomous and therefore could not be compared directly. Inflammation would be reflected in symptoms and abnormal Hgb and or ESR, while serology would be expected to be negative. This

TABLE 3. Sensitivity, specificity, positive and negative predictive value of serology, abnormal laboratory tests, and rectal bleeding for screening children with inflammatory bowel disease (%)

Screening test combinations for the diagnosis of IBD	ASCA+ and/or pANCA+	Abnormal Hgb/ESR	Abnormal Hgb/ESR and rectal bleeding	Positive serology and/or abnormal Hgb/ESR	Positive serology and/or abnormal Hgb/ESR and rectal bleeding
Sensitivity	68	62	86	83	89
Specificity	92	75	67	70	62
Predictive value of positive test	92	79	79	81	78
Predictive value of negative test	66	58	76	74	79

ASCA, anti-*Saccharomyces cerevisiae* antibody; pANCA, perinuclear antineutrophil cytoplasmic antibody; Hgb, hemoglobin; ESR, erythrocyte sedimentation rate.

may be a valid method of discerning IBD from other forms of inflammation; however, a colonoscopic examination would still be required for diagnosis. Serology was negative in this group, apart from one child with indeterminate colitis who was pANCA-positive and a second who had a borderline positive ASCA at initial investigation but was negative when repeated within 2 weeks.

RESULTS

The most common symptoms at presentation are listed in Table 2. Less frequent symptoms included nausea, vomiting, fever, fatigue or lethargy, and pallor. Visible bleeding per rectum was the most discriminating symptom for individuals with IBD compared with the group without IBD ($p < 0.05$); also, there was a significant difference for diarrhea ($p < 0.05$) but not abdominal pain ($p = 0.35$).

The number of individuals with positive serology in each diagnostic category are listed in Table 2. Both individuals who were ASCA positive but without IBD (false positives) were investigated with upper endoscopy and small bowel follow-through. Results of ASCA in CD included IgA, 7 of 39, IgG, 3 of 39, and both, 8 of 39. CD limited to the small bowel was present in 5 of 39. Four of 5 had abnormal laboratory tests and abnormal serology. Of the serology-positive children with IBD, 3 of 61 were both ASCA- and pANCA-positive, 1 of 3 with CD and 2 of 3 with CUC. There was a significant difference between the proportion with a positive pANCA (42/51) and the combination of Hgb and ESR (30/51) for CUC ($p < 0.05$). The proportion of ASCA-positive children with CD (18/39) was less than those screened with the combination of Hgb and ESR (26/39). The difference was not statistically significant ($p = 0.27$).

Sensitivity and Specificity

The sensitivity and specificity, respectively, for CUC were 3.9%, 99% (pANCA⁺, ASCA⁺), 77%, 96% (pANCA⁺, ASCA⁻), 0%, 81% (pANCA⁻, ASCA⁺), 22%, 78% (pANCA⁻, ASCA⁻). For CD, the respective values were 2.6%, 98% (pANCA⁺, ASCA⁺), 2.6%, 63% (pANCA⁺, ASCA⁻), 44%, 97% (pANCA⁻, ASCA⁺), 56%, 60% (pANCA⁻, ASCA⁻). Table 3 shows the sensitivities and specificities of serology and routine laboratory tests in screening for IBD. The highest sensitivity was obtained with the combination of Hgb, ESR, and rectal bleeding (86%), while the specificity was highest with serology (combined pANCA and ASCA) (92%). The potential value of serology in screening symptomatic children is shown in Table 4. Three of 90 children

would have been screened positive by the addition of serology to routine laboratory tests.

DISCUSSION

An ideal screening test should be disease specific and sensitive, simple to apply, minimally invasive, inexpensive, and individually reproducible, with respect to patients and laboratories (2). Combined serology with pANCA and ASCA showed excellent specificity for the diagnosis of IBD. The combination of routine laboratory tests and presence of rectal bleeding had better sensitivity than serology (Table 3). On the other hand, the specificity of routine tests was poor in comparison to serology, and the addition of other nonspecific markers while improving sensitivity may have resulted in a further loss of specificity. The short falls of serology were related to ASCA. Our data indicated that one-half of children with CD were not identified by serology in clinical practice. The percentage of CD cases screened positive with ASCA were less than those screened positive for IBD with routine laboratory tests. Furthermore, children with CD who were ASCA positive also were positive with routine laboratory tests. Taking in to consideration the presence of rectal bleeding, routine laboratory tests, and serology, only 76% of children with CD in our series would have been identified as possibly having IBD prior to an endoscopic procedure. It is clear from our data that negative results for any or all these laboratory tests should not preclude an endoscopic procedure in symptomatic children.

Other than diagnostic screening, the role of routine laboratory tests is different than serology in the management of IBD. A raised ESR correlates with the degree of intestinal inflammation making it suitable for monitoring treatment (2,7,8). In contrast, serology has no role in monitoring disease. Treatment does not alter the expression of pANCA, and in the studies so far, the indications are that ASCA behaves similarly (3). Some authors have

TABLE 4. The effect of adding serology to routine screening tests in identifying patients for an endoscopic procedure

	Inflammatory bowel disease n = 90	No inflammatory bowel disease n = 61
Visible rectal bleeding	61	5
+ Additional positives from screening with Hgb and ESR	16	20
+ Additional positives from screening with serology	3	3

Hgb, hemoglobin; ESR, erythrocyte sedimentation rate.

suggested that ASCA titers may be reduced with treatment (7). Anti-*Saccharomyces cerevisiae* antibody positivity is related to small bowel CD, a feature not adequately examined in our study due to the small number of patients with disease limited to the small bowel (3,7). It also is expressed by unaffected first-degree family members of CD patients (9). A similar proportion (20%) of healthy relatives of CD patients have been reported as having increased intestinal permeability (10). Antibodies to a glycoprotein antigen of *Saccharomyces cerevisiae* were also reported in 6 of 10 controls in comparison with 8 of 10 Crohn's cases in one study (11). This may be a reflection on the lack of standardized ASCA serology (6).

There remains an ongoing debate as to the role of serology in the management of IBD (12). Indeterminate colitis is suggested to be an area where serology for IBD may prove to be useful (10,13). In our series, individuals with indeterminate colitis were serology negative. This may be a result of bias, as physicians were not blinded to the results of serology. The proportion of individuals with indeterminate colitis was very similar to those of previous studies (5,7). Incidentally, in our series, the assignment of a specific diagnosis to individuals from the group with indeterminate colitis would have reduced the sensitivity of serology. Positive pANCA in 83% of individuals with CUC in our series was similar to previous reports (3,5,7). This suggests that the population of children screened with serology in our clinical practice was similar to the cohorts in previous studies (5,7,14). In contrast to some previous reports (6), ASCA was positive in fewer than 50% of CD cases. A study in the Greek adult population also reported limited sensitivity of ASCA for CD (14). The authors postulated that the phenotype of their population may be different than that of other studies. A more recent reviewer indicated that this finding was a limitation of the test (15). The sensitivity of the serology tests from Prometheus Laboratories was analyzed in a recent study (16). The authors examined how altering the cut-off levels for the serological assays improved sensitivity for the initial screening tests (the first step). The modifications also resulted in reduced specificity. Our results showed that the sensitivity of screening was improved by combining serology from Prometheus Laboratories with conventional screening tests from 86% to 89% (Table 3), and would have resulted in 3 of 90 additional cases (Table 4) screened positive before an endoscopic procedure was performed. As a diagnostic tool, serology may therefore add little to existing laboratory tests and clinical history. Our findings are in keeping with a recent editorial review (12). There also is a significant cost attached to combined

serology with ASCA and pANCA, which at present are only performed in a few centers. Results may therefore only become available after endoscopic evaluation has been completed. In the clinical setting where Hgb and ESR are usually performed early in the investigation of IBD, symptomatic children who screen positive with routine laboratory tests should undergo an endoscopic procedure regardless of serology results (15,16). Furthermore in our study only 80 of 90 individuals were identified before endoscopy using all parameters, indicating that a clear detailed history and a high index of suspicion are the most valuable part of the screening process.

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TITLE: Method for detecting enteric disease

Brief Summary Text (20):

Diarrhea may be caused by factors other than invasive enteric pathogens, and it is particularly useful for the treating physician to know whether, in the absence of various pathogens that are typically assayed, the diarrhea is associated with an inflammatory condition of the intestines or has other, non-inflammatory--and hence, generally, non-pathogenic--causes. Lactoferrin is an iron-binding bactericidal protein contained in granules in polymorphonuclear (PMN) leukocytes and is found in intestinal secretions, as well as in other secretions. Since the PMNs increase rapidly in number in response to an infection, the number of lactoferrin granules also increases. Studies have shown that the presence of fecal lactoferrin is a reliable general indicator of inflammation in the intestinal tract. See Choi et al.; To Culture or Not to Culture: Fecal Lactoferrin Screening for Inflammatory Bacterial Diarrhea, in: Journal of Clinical Microbiology, April 1996, p. 928-932. Because lactoferrin is also found in breast milk, fecal lactoferrin found in breast-fed infants is not an effective indicator of an inflammatory intestinal condition, as the presence of lactoferrin from breast milk will lead to false positives. Nevertheless, a method for detecting multiple enteric foodborne pathogens that includes a general indicator for an inflammatory condition of the intestines will provide the physician who is treating a patient other than a breast-fed infant with valuable information.

Other Reference Publication (14):

Sugi et al., "Fecal Lactoferrin as a Marker for Disease Activity in Inflammatory Bowel Disease: Comparison with Other Neutrophil-derived Proteins", The American J. of Gastroenterology, vol. 91, pp. 927-934, 1996, USA.

Other Reference Publication (17):

Fine, et al., "Utility of a Rapid Fecal Latex Agglutination Test Detecting the Neutrophil Protein, Lactoferrin, for Diagnosing Inflammatory Causes of Chronic Diarrhea", American J. of Gastroenterology, vol. 93, No. 8, 1998, pp. 1300-1305. USA.

ASA
fecal sample

DOCUMENT-IDENTIFIER: US 20010036639 A1

TITLE: Method for diagnosing immunologic food sensitivity

CLAIMS:

1. A method for diagnosing an immunologic food sensitivity comprising the steps of: collecting a fecal sample; screening the fecal sample to detect the presence of an antibody to a particular food substance; and diagnosing an immunologic food sensitivity based on the presence of the antibody.
2. The method of claim 1 further comprising the step of concentrating the fecal sample to obtain a testing portion after said collecting step and wherein said testing portion is the sample in said screening step.
4. The method of claim 2 further comprising the step of homogenizing the fecal sample prior to said concentrating step.
8. The method of claim 2 wherein said concentrating step comprises the steps of: centrifuging the fecal sample; removing a supernatant portion from the centrifuged fecal sample; and using the supernatant portion as the testing portion.
12. The method of claim 2 wherein said concentrating step comprises the steps of: freeze-drying the fecal sample to a solid material; and reconstituting the solid material with water to form a reconstituted testing portion.
14. The method of claim 2 wherein said fecal sample contains more than about 90% water in its excreted state and wherein said concentrating step comprises the steps of: freeze-drying the fecal sample to a solid material; and reconstituting the solid material with water to form a reconstituted testing portion.
21. The method of claim 19 wherein said yeast is Saccharomyces cerevisiae.
43. The method of claim 33 wherein said sample is a fecal sample.
44. The method of claim 43 further comprising the step of concentrating said fecal sample to obtain a testing portion prior to said screening step and wherein said testing portion is the sample in said screening step.
45. The method of claim 44 further comprising the step of homogenizing the fecal sample prior to concentrating said sample.
49. A method for diagnosing an immunologic drug sensitivity comprising the steps of: collecting a fecal sample; screening the fecal sample to detect the presence of an antibody to a particular drug substance; and diagnosing an immunologic drug sensitivity based on the presence of the antibody.
50. The method of claim 49 further comprising the step of concentrating the fecal sample to obtain a testing portion prior to said screening step and wherein said testing portion is the sample in said screening step.
52. The method of claim 50 wherein said concentrating step comprises the steps of: centrifuging the fecal sample; removing a supernatant portion from the centrifuged fecal sample; and using the supernatant portion as the testing portion.

54. The method of claim 50 wherein said concentrating step comprises the steps of: freeze-drying the fecal sample to a solid material; and reconstituting the solid material with water to form a reconstituted testing portion.

56. The method of claim 55 further comprising the following steps: collecting a fecal sample; screening the fecal sample to detect the presence of an antibody to a particular food substance; and confirming diagnosis of an immunologic food sensitivity based on the presence of the antibody.

57. The method of claim 56 further comprising the step of concentrating the fecal sample to obtain a testing portion and wherein said testing portion is the sample in said screening step.

58. The method of claim 57 further comprising the step of homogenizing the fecal sample prior to concentrating said sample.

60. The method of claim 57 wherein said concentrating step comprises the steps of: centrifuging the fecal sample; removing a supernatant portion from the centrifuged fecal sample; and using the supernatant portion as the testing portion.

62. The method of claim 57 wherein said concentrating step comprises the steps of: freeze-drying the fecal sample to a solid material; and reconstituting the solid material with water to form a reconstituted testing portion.

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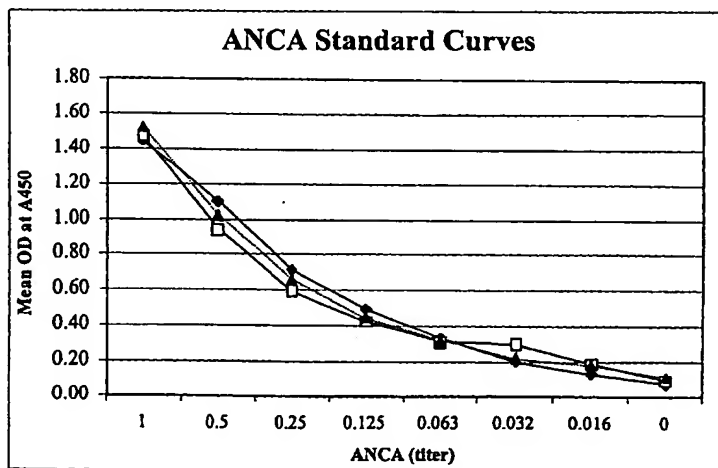
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(54) Title: METHOD FOR DISTINGUISHING ULCERATIVE COLITIS FROM CROHN'S DISEASE BY DETECTING THE PRESENCE OF FECAL ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA)



(57) Abstract: A method and apparatus for the differentiation of ulcerative colitis from Crohn's disease and other gastrointestinal illnesses using the presence of anti-neutrophil cytoplasmic antibodies (ANCA) as a marker of ulcerative colitis is described. The apparatus consists of either a qualitative enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobulins for the measurement of total endogenous ANCA in a human sample. The method and apparatus can be used by healthcare providers to distinguish ulcerative colitis from Crohn's disease and other gastrointestinal.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**METHOD FOR DISTINGUISHING ULCERATIVE COLITIS FROM
CROHN'S DISEASE BY DETECTING THE PRESENCE OF FECAL
ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA)**

BACKGROUND OF THE INVENTION

5 This invention relates to non-invasive methods for differentiating clinical subtypes of Inflammatory Bowel Disease, namely Crohn's disease (CD) and ulcerative colitis (UC). More specifically, this invention relates to a method and apparatus for aiding in the differentiation of Crohn's disease from ulcerative colitis by determining the presence of anti-neutrophil cytoplasmic antibodies
10 (ANCA), wherein the presence of ANCA is indicative of ulcerative colitis. In addition, the presence of fecal ANCA may be used to differentiate ulcerative colitis from other gastrointestinal illnesses such as Irritable Bowel Syndrome.

 An estimated 1 million Americans suffer from Inflammatory Bowel Disease (IBD). IBD is characterized by a chronic inflammatory response
15 that results in histologic damage to the intestinal lining. Crohn's disease may involve the entire gastrointestinal tract and include inflammation extending into the transmural mucosa, whereas ulcerative colitis affects solely the large bowel and includes inflammation of the innermost lining. These two distinct diseases require a rapid differential diagnosis for optimal treatment. Conventional
20 methods utilizing multiple endoscopy examinations and histological analysis may take years to confirm a diagnosis. U.S. Patent No. 6,218,120 discloses a method of determining the presence of serum ANCA as a marker to diagnose IBD. However, it does not disclose a method for diagnosing ulcerative colitis in a patient diagnosed with IBD. Further, the method does not disclose testing human
25 feces for the presence of ANCA.

 Accordingly, there remains a need in the diagnostic industry for a non-invasive method of differentially diagnosing ulcerative colitis from Crohn's disease or other gastrointestinal illnesses.

SUMMARY OF THE INVENTION

30 Accordingly, in one of its aspects, the present invention provides non-invasive methods for differentiating between diagnoses of ulcerative colitis and Crohn's disease.

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In another of its aspects, the present invention provides methods for differentiating between ulcerative colitis and Crohn's disease wherein the presence of fecal ANCA is used as a marker for ulcerative colitis.

5 In a further aspect, the present invention provides immunoassays, e.g., and enzyme-linked immunoassays, that utilize antibodies specific to human immunoglobulins for the measurement of total endogenous ANCA in human feces.

10 In yet another of its aspects, the present invention provides methods differentially diagnosing ulcerative colitis from other gastrointestinal illnesses such as Irritable Bowel Syndrome (IBS). In still another of its aspects, the present invention provides methods for diagnosing ulcerative colitis wherein the presence of ANCA is used as a marker for ulcerative colitis.

15 According to the present invention, the foregoing and other aspects are achieved by a non-invasive method for aiding in the differentiation of ulcerative colitis from Crohn's disease in a patient presenting with IBD. In the method of the present invention, fecal ANCA are used as a marker and the presence of ANCA indicates a differential diagnosis of ulcerative colitis. This rapid diagnosis may then be used by healthcare professionals to prescribe proper treatment.

20 Aspects of the present invention are further achieved by immunoassays that utilize antibodies specific to human immunoglobulins for the measurement of total endogenous ANCA in human feces.

25 Additional aspects of the invention, together with the advantages and novel features appurtenant thereto, will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned from the practice of the invention. The objects and advantages of the invention may be realized and attained by means, instrumentality's and combinations particularly pointed out in the appended claims.

30 BRIEF DESCRIPTION OF THE VIEW OF THE DRAWING

Fig. 1 is a graphical representation of a standard curve of anti-neutrophil cytoplasmic antibodies in accordance with an embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to non-invasive methods for differentiating between ulcerative colitis and Crohn's disease using the presence of fecal ANCA as an indicator of ulcerative colitis. The present invention also is directed to a method for differentiating between ulcerative colitis and other gastrointestinal illnesses such as IBS. The present invention is further directed to immunoassays that utilize antibodies specific to human immunoglobulins for the measurement of total endogenous ANCA in human feces. The particular embodiments described herein are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its scope.

ANCA specific immunoassays may be used to differentiate ulcerative colitis and indeterminate colitis from Crohn's disease by measurement of the presence of total endogenous ANCA. In addition to fecal matter, a sample of whole blood, serum, plasma or other bodily fluid or tissue may be tested for ANCA to diagnose ulcerative colitis. This differential diagnosis may then be used by healthcare professionals for determining optimal treatment. A qualitative immunoassay, such as a later flow dipstick that utilizes both monoclonal and polyclonal antibodies to endogenous human ANCA to indicate the presence of ulcerative colitis.

In the qualitative immunoassay, the fecal or bodily sample is diluted 10 fold and added to a well containing immobilized neutrophilic antigens. If endogenous fecal ANCA is present, it will bind to the neutrophilic antigens during an incubation step at 37°C. Following the incubation, polyvalent antibodies to human immunoglobulin coupled to an enzyme, such as a horseradish peroxidase enzyme, (conjugate) is added and allowed to bind to captured ANCA. Unbound conjugate is then washed from the well and one component substrate (e.g., tetramethylbenzidine and hydrogen peroxide) is added for color development. Following the substrate incubation, 0.1M sulfuric acid is added to stop the reaction and the optical density (OD) is obtained spectrophotometrically at 450 nm.

In a clinical study, a total of 98 IBD patients were enrolled and comprised 51% males and 49% females with an age range of 0 to 69 years. The approximate 1 to 1 ratio is similar to the ratio observed in IBD patient populations. The IBS patient group had an age range of 5 to 39 years with 57% males and 43% females. The healthy controls were 55% male and 45% female and comprised the age range of 20 to 79 years. Individual numbers for each age group are shown in Table 1.

TABLE 1. Summary of patient population.

Summary of Clinical Histories (N=116)	Total Subjects
Total number of IBD patients	98
No. Males	50
No. Females	48
Total number of patients with Crohn's Disease	47
No. Males	26
No. Females	21
Total number of patients with ulcerative colitis	51
No. Males	24
No. Females	27
Total number of patients with irritable bowel syndrome	7
No. Males	4
No. Females	3
Total number of healthy persons	11
No. Males	6
No. Females	5

10

There were 51 ulcerative colitis (UC) patients, 47 Crohn's disease (CD) patients, 7 irritable bowel patients (IBS), and 11 healthy (H) adults recruited for the study. Fecal specimens were collected from each enrolled patient and stored at -70°C until tested. Specimen consistency ranged from solid to liquid. The level of fecal ANCA was determined using the qualitative ANCA ELISA as previously described. Disease activity was defined using elevated fecal lactoferrin as an indicator of intestinal inflammation. A dilution of 1:10 was used in the qualitative ELISA test and results were reported as positive (absorbance values ≥ 0.140) or negative (absorbance values < 0.140). The mean

15

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optical densities, standard deviation and P values (two-tailed student T-test with unequal variance) were determined for the ANCA positive ulcerative colitis patients. Of the 26 patients that tested positive for fecal ANCA, there were four patients had Crohn's Disease, 21 had ulcerative colitis and one patient was healthy. ANCA-positive ulcerative colitis showed a mean \pm SD OD₄₅₀ of 0.311 ± 0.166 . The mean optical density for the ulcerative colitis patients was significantly different from IBS and healthy persons (p value < 0.0005). A summary of the statistical analysis is listed in Table 2.

TABLE 2. Summary of the mean, standard deviation and P values for qualitative ELISA test Optical Densities

Group ID	Number	Mean Optical Density	Standard Deviation	Optical Density Range	P values
ANCA + UC	21	0.311	0.166	0.141-0.804	UC vs CD p<0.5
ANCA + CD	4	0.209	0.115	0.141-0.381	UC vs CD, IBS, H p<0.0005
IBS	7	0.078	0.027	0.047-0.121	UC vs CD, IBS p<0.005
Healthy	11	0.071	0.041	0.039-0.104	UC vs IBS, H p<0.0005

In the group of patients with IBD, there were 47 with Crohn's disease and 51 with ulcerative colitis. In the ulcerative colitis group, 41% were positive. In the Crohn's disease group, a total of 9% patients were positive using the qualitative ELISA test. Of the 11 healthy persons, 1 was positive and all 7 IBS patients were negative by the qualitative ELISA test. A summary of positive results for the qualitative ELISA test are shown in Table 3 and individual results are listed in Table 4 and Table 5.

TABLE 3. Summary of positive results for Crohn's disease, ulcerative colitis, and IBS

Total Assessments N=116	Total	Fecal ANCA Positive	Fecal ANCA Negative
Total IBD (Crohn's disease and ulcerative colitis)	98	26% (25)	75% (73)
Total Crohn's Disease	47	9% (4)	91% (43)
Total Ulcerative Colitis	51	41% (21)	59% (30)
Total IBS	7	0	7
Total Healthy Persons	11	9%(1)	91%(10)

5 When distinguishing ulcerative colitis from Crohn's disease, the qualitative ELISA test exhibited a sensitivity of 41% and specificity of 92%. The predictive positive and negative values were 84% and 59%, respectively, and the correlation was 65% (Table 4).

10 **TABLE 4. Statistical evaluation using the qualitative ELISA test to distinguish Crohn's disease from ulcerative colitis**

N=98	Ulcerative colitis	Crohn's disease
ANCA positive	21	4
ANCA negative	30	43

Sensitivity	41%
Specificity	92%
Predictive Positive Value	84%
Predictive Negative Value	59%
Correlation	65%

15 When distinguishing ulcerative colitis from irritable bowel syndrome and healthy persons, the qualitative ELISA test exhibited a sensitivity of 41% and a specificity of 92%. The predictive positive and negative values

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were 81% and 67%, respectively, and the correlation was 70% as shown in Table 5.

5 **TABLE 5. Statistical evaluation using the qualitative ELISA test to distinguish ulcerative colitis from Crohn's disease, irritable bowel syndrome and healthy persons**

N=116	Ulcerative colitis	Crohn's disease IBS/Healthy
ANCA positive	21	5
ANCA negative	30	60

Sensitivity	41%
Specificity	92%
Predictive Positive Value	81%
Predictive Negative Value	67%
Correlation	70%

10 The sensitivity of the qualitative ELISA test was determined using serial two fold dilutions of human ANCA positive serum. For the analysis, standard curves were generated using the sample diluent. The test was consistently positive to a titer of 0.063 as determined by a cutoff absorbance value of ≥ 0.200 . Individual results are shown below in Table 6 and standard curves are shown in FIG. 1.

15 **TABLE 6. Standard curves generated using qualitative ELISA test (cut-offs are in bold)**

Human ANCA Serum	Test 1	Test 2	Test 3	Mean	Std Dev
1.000 (Neat)	1.441	1.469	1.525	1.478	0.043
0.500	1.098	0.941	1.014	1.018	0.079
0.250	0.717	0.595	0.666	0.659	0.061
0.125	0.492	0.428	0.444	0.455	0.033
0.063	0.327	0.303	0.320	0.317	0.012
0.032	0.196	0.295	0.221	0.237	0.051
0.016	0.132	0.184	0.179	0.165	0.029
Diluent	0.067	0.093	0.109	0.090	0.021

20 Table 7, below, contains the clinical data and test results for patients with ulcerative colitis that participated in the study. Table 8, below, contains the clinical data and test results for patients with Crohn's disease that participated in the study. Table 9, below, contains the clinical data and test

results for patients with irritable bowel syndrome that participated in the study. Table 10, below, contains the clinical data and test results for health patients that participated in the study.

TABLE 7. Clinical data and ELISA results for ulcerative colitis patients.

5

Patient ID	Sex	Age Range	Disease	Disease Activity	ELISA OD ₄₅₀	ELISA Result
UC1	F	10-19	UC	INACTIVE	0.053	NEGATIVE
UC2	F	5-9	UC	INACTIVE	0.107	NEGATIVE
UC3	F	5-9	UC	ACTIVE	0.058	NEGATIVE
UC4	M	10-19	UC	INACTIVE	0.048	NEGATIVE
UC5	M	10-19	UC	ACTIVE	0.512	POSITIVE
UC6	F	10-19	UC	ACTIVE	0.061	NEGATIVE
UC7	M	5-9	UC	ACTIVE	0.211	POSITIVE
UC8	M	10-19	UC	ACTIVE	0.106	NEGATIVE
UC9	M	10-19	UC	INACTIVE	0.804	POSITIVE
UC10	M	10-19	UC	ACTIVE	0.091	NEGATIVE
UC11	F	10-19	UC	ACTIVE	0.169	POSITIVE
UC12	F	10-19	UC	ACTIVE	0.209	POSITIVE
UC13	F	10-19	UC	ACTIVE	0.351	POSITIVE
UC14	F	10-19	UC	ACTIVE	0.198	POSITIVE
UC15	F	5-9	UC	ACTIVE	0.098	NEGATIVE
UC16	F	5-9	UC	ACTIVE	0.050	NEGATIVE
UC17	F	10-19	UC	ACTIVE	0.091	NEGATIVE
UC18	M	10-19	UC	ACTIVE	0.603	POSITIVE
UC19	M	10-19	UC	ACTIVE	0.091	NEGATIVE
UC20	F	10-19	UC	ACTIVE	0.142	POSITIVE
UC21	M	10-19	UC	ACTIVE	0.074	NEGATIVE
UC22	F	10-19	UC	ACTIVE	0.105	NEGATIVE
UC23	M	10-19	UC	INACTIVE	0.256	POSITIVE
UC24	F	0-4	UC	ACTIVE	0.308	POSITIVE
UC25	F	5-9	UC	ACTIVE	0.072	NEGATIVE
UC26	M	10-19	UC	INACTIVE	0.237	POSITIVE
UC27	M	10-19	UC	ACTIVE	0.048	NEGATIVE
UC28	M	10-19	UC	ACTIVE	0.049	NEGATIVE
UC29	M	10-19	UC	ACTIVE	0.059	NEGATIVE
UC30	F	10-19	UC	INACTIVE	0.047	NEGATIVE
UC31	M	10-19	UC	ACTIVE	0.055	NEGATIVE
UC32	M	10-19	UC	INACTIVE	0.044	NEGATIVE
UC33	F	10-19	UC	ACTIVE	0.043	NEGATIVE

UC34	M	5-9	UC	ACTIVE	0.046	NEGATIVE
UC35	M	10-18	UC	INACTIVE	0.043	NEGATIVE
UC36	M	10-17	UC	INACTIVE	0.040	NEGATIVE
UC37	F	10-19	UC	ACTIVE	0.047	NEGATIVE
UC38	F	0-4	UC	ACTIVE	0.049	NEGATIVE
UC39	F	5-9	UC	INACTIVE	0.363	POSITIVE
UC40	F	10-19	UC	INACTIVE	0.046	NEGATIVE
UC41	M	10-19	UC	ACTIVE	0.118	NEGATIVE
UC42	F	50-59	UC	ACTIVE	0.230	POSITIVE
UC43	M	10-19	UC	ACTIVE	0.051	NEGATIVE
UC44	F	30-39	UC	ACTIVE	0.060	NEGATIVE
UC45	F	50-59	UC	ACTIVE	0.465	POSITIVE
UC46	M	50-59	UC	ACTIVE	0.274	POSITIVE
UC47	F	30-39	UC	ACTIVE	0.141	POSITIVE
UC48	M	60-69	UC	ACTIVE	0.184	POSITIVE
UC49	F	40-49	UC	ACTIVE	0.397	POSITIVE
UC50	F	40-49	UC	ACTIVE	0.337	POSITIVE
UC51	M	30-39	UC	ACTIVE	0.143	POSITIVE

TABLE 8. Clinical data and ELISA results for Crohn's disease patients.

Patient ID	Sex	Age Range	Disease	Disease Activity	ELISA OD ₄₅₀	ELISA Result
CD1	M	10-19	CD	ACTIVE	0.050	NEGATIVE
CD2	M	10-19	CD	ACTIVE	0.113	NEGATIVE
CD3	M	10-19	CD	ACTIVE	0.050	NEGATIVE
CD4	F	10-19	CD	ACTIVE	0.381	POSITIVE
CD5	F	10-19	CD	ACTIVE	0.058	NEGATIVE
CD6	M	10-19	CD	INACTIVE	0.068	NEGATIVE
CD7	M	10-19	CD	ACTIVE	0.066	NEGATIVE
CD8	M	5-9	CD	ACTIVE	0.059	NEGATIVE
CD9	F	10-19	CD	ACTIVE	0.059	NEGATIVE
CD10	F	10-19	CD	ACTIVE	0.065	NEGATIVE
CD11	F	10-19	CD	INACTIVE	0.055	NEGATIVE
CD12	M	10-19	CD	INACTIVE	0.071	NEGATIVE
CD13	F	10-19	CD	ACTIVE	0.065	NEGATIVE
CD14	M	10-19	CD	ACTIVE	0.098	NEGATIVE
CD15	F	10-19	CD	ACTIVE	0.099	NEGATIVE
CD16	M	10-19	CD	ACTIVE	0.166	POSITIVE
CD17	F	10-19	CD	ACTIVE	0.147	POSITIVE
CD18	M	10-19	CD	ACTIVE	0.057	NEGATIVE

CD19	F	10-19	CD	ACTIVE	0.084	NEGATIVE
CD20	M	10-19	CD	ACTIVE	0.053	NEGATIVE
CD21	F	10-19	CD	ACTIVE	0.074	NEGATIVE
CD22	M	10-19	CD	ACTIVE	0.054	NEGATIVE
CD23	M	0-5	CD	ACTIVE	0.055	NEGATIVE
CD24	M	10-19	CD	ACTIVE	0.067	NEGATIVE
CD25	M	10-19	CD	ACTIVE	0.099	NEGATIVE
CD26	M	5-9	CD	ACTIVE	0.086	NEGATIVE
CD27	F	10-19	CD	ACTIVE	0.043	NEGATIVE
CD28	F	10-19	CD	ACTIVE	0.064	NEGATIVE
CD29	M	5-9	CD	INACTIVE	0.039	NEGATIVE
CD30	M	10-19	CD	ACTIVE	0.071	NEGATIVE
CD31	F	10-15	CD	ACTIVE	0.109	NEGATIVE
CD32	M	10-19	CD	INACTIVE	0.057	NEGATIVE
CD33	M	10-19	CD	ACTIVE	0.141	POSITIVE
CD34	M	10-19	CD	INACTIVE	0.045	NEGATIVE
CD35	F	10-19	CD	ACTIVE	0.051	NEGATIVE
CD36	F	10-19	CD	ACTIVE	0.132	NEGATIVE
CD37	F	10-19	CD	INACTIVE	0.046	NEGATIVE
CD38	M	10-19	CD	ACTIVE	0.057	NEGATIVE
CD39	F	20-29	CD	INACTIVE	0.051	NEGATIVE
CD40	F	20-29	CD	ACTIVE	0.053	NEGATIVE
CD41	M	50-59	CD	ACTIVE	0.060	NEGATIVE
CD42	F	50-59	CD	ACTIVE	0.062	NEGATIVE
CD43	M	20-29	CD	ACTIVE	0.056	NEGATIVE
CD44	F	60-69	CD	ACTIVE	0.130	NEGATIVE
CD45	M	60-69	CD	ACTIVE	0.078	NEGATIVE
CD46	F	40-49	CD	ACTIVE	0.116	NEGATIVE
CD47	M	60-69	CD	ACTIVE	0.057	NEGATIVE

TABLE 9. Clinical data and ELISA results for Irritable bowel syndrome patients.

Patient ID	Sex	Age Range	Disease	ELISA OD ₄₅₀	ELISA Results
IBS1	F	10-19	IBS	0.056	NEGATIVE
IBS2	M	10-19	IBS	0.047	NEGATIVE
IBS3	M	5-9	IBS	0.099	NEGATIVE
IBS4	M	10-19	IBS	0.068	NEGATIVE
IBS5	M	10-19	IBS	0.092	NEGATIVE
IBS6	F	20-29	IBS	0.121	NEGATIVE
IBS7	F	30-39	IBS	0.064	NEGATIVE

TABLE 10. Clinical data and ELISA results for healthy persons.

Subject ID	Sex	Age Range	ELISA OD ₄₅₀	ELISA Results
D1	F	40-49	0.087	NEGATIVE
D2	M	20-29	0.078	NEGATIVE
D5	M	20-29	0.178	POSITIVE
D15	M	50-59	0.041	NEGATIVE
D17	M	50-59	0.039	NEGATIVE
D18	F	40-49	0.069	NEGATIVE
D19	F	60-69	0.050	NEGATIVE
D20	M	70-79	0.039	NEGATIVE
D21	F	70-79	0.104	NEGATIVE
D22	M	60-69	0.045	NEGATIVE
D24	F	50-59	0.054	NEGATIVE

In summary, the present invention is directed to non-invasive methods for aiding in the differentiation of ulcerative colitis from Crohn's disease by determining the presence of ANCA as a marker of ulcerative colitis. The present invention is further drawn to immunoassays, e.g., qualitative enzyme-linked immunoassays, that utilize antibodies specific to human immunoglobulins for the measurement of total endogenous ANCA in human feces. The present invention has been described in relation to particular embodiments which are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its scope.

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From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects hereinabove set forth together with other advantages which are obvious and which are inherent to the method.

It will be understood that certain features and subcombinations are
5 of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims.

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CLAIMS

Having thus described the invention, what is claimed is:

1. A method for testing a fecal sample, the method
5 comprising: obtaining a fecal sample from a person; and determining whether anti-neutrophil cytoplasmic antibodies are present in the sample.
2. The method of claim 1, wherein if the sample contains anti-neutrophil cytoplasmic antibodies, a diagnosis of ulcerative colitis may be substantially concluded.
- 10 3. The method of claim 2, wherein the presence of anti-neutrophil cytoplasmic antibodies is used to aid in the differentiation of ulcerative colitis from Crohn's disease.
4. The method of claim 2, wherein the presence of anti-neutrophil cytoplasmic antibodies is used to aid in the differentiation of ulcerative
15 colitis from other gastrointestinal illnesses.
5. The method of claim 4, wherein the other gastrointestinal illness is irritable bowel syndrome.
6. The method as recited in claim 1, wherein the endogenous anti-neutrophil cytoplasmic antibodies comprise the total anti-neutrophil
20 cytoplasmic antibodies.
7. The method as recited in claim 1, further comprising: diluting the fecal sample.
8. The method as recited in claim 7, further comprising: contacting the sample with neutrophil cytoplasmic antigens to create a treated
25 sample.
9. The method as recited in claim 8, further comprising: contacting the treated sample with polyvalent antibodies to human immunoglobulin to create a readable sample.

10. The method as recited in claim 9, further comprising:
determining an optical density of the readable sample at 450 nm, wherein the
optical density corresponds to a level of endogenous anti-neutrophil cytoplasmic
antibodies in the sample.

5 11. A diagnostic assay for diagnosing ulcerative colitis by
determining the endogenous anti-neutrophil cytoplasmic antibodies, the assay
comprising: obtaining a human fecal sample; diluting the fecal sample;
contacting the sample with neutrophil cytoplasmic antigens to create a treated
sample; contacting the treated sample with polyvalent antibodies to human
10 immunoglobulin to create a readable sample; determining the optical density of
the readable sample at 450 nm.

12. The diagnostic assay as recited in claim 11, wherein if the
readable sample contains endogenous anti-neutrophil cytoplasmic antibodies, a
diagnosis of ulcerative colitis is substantially concluded.

15 13. The diagnostic assay as recited in claim 12, wherein the
antibodies are one of IgG, IgE, IgM, IgD, IgA_{sec}, IgA, and combinations thereof.

14. The diagnostic assay as recited in claim 1, wherein the
assay comprises one of an enzyme-linked immunoassay and a lateral flow
membrane test.

20 15. A kit for diagnosing ulcerative colitis by testing a fecal
sample from a person to be diagnosed, the kit comprising: one or more
microassay plates, each the plate containing neutrophil cytoplasmic antigens;
polyvalent antibodies to human immunoglobulin; and enzyme substrate for color
development.

25 16. The kit as recited in claim 15, further comprising a stop
solution for quenching the reaction.

17. A method for screening for ulcerative colitis, the method comprising: obtaining a sample from a person; determining whether anti-neutrophil cytoplasmic antibodies are present in the sample; and if so, a diagnosis of ulcerative colitis may be substantially concluded.

5 18. The method of claim 17, wherein the presence of anti-neutrophil cytoplasmic antibodies is used to aid in the differentiation of ulcerative colitis from Crohn's disease.

 19. The method of claim 17, wherein the presence of anti-neutrophil cytoplasmic antibodies is used to aid in the differentiation of ulcerative
10 colitis from other gastrointestinal illnesses.

 20. The method as recited in claim 17, wherein the endogenous anti-neutrophil cytoplasmic antibodies comprise the total anti-neutrophil cytoplasmic antibodies.

 21. The method as recited in claim 17, further comprising:
15 diluting the sample.

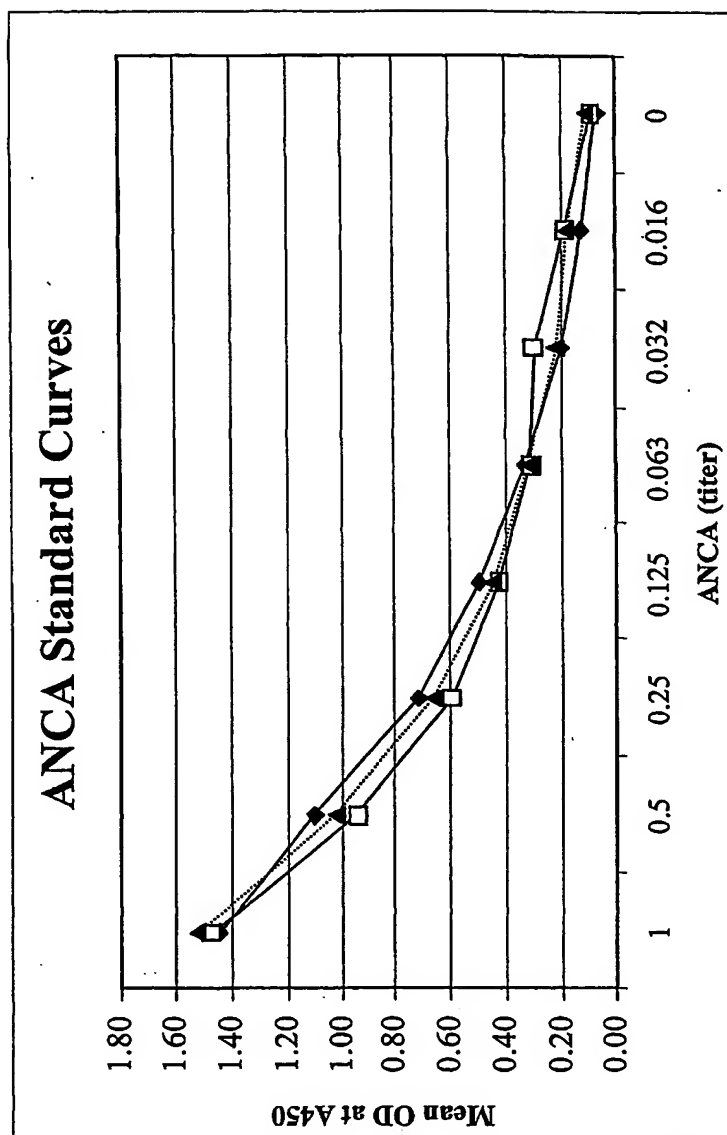
 22. The method as recited in claim 21, further comprising: contacting the sample with neutrophil cytoplasmic antigens to create a treated sample.

 23. The method as recited in claim 22, further comprising:
20 contacting the treated sample with polyvalent antibodies to human immunoglobulin to create a readable sample.

 24. The method as recited in claim 23, further comprising: determining an optical density of the readable sample at 450 nm, wherein the optical density corresponds to a level of endogenous anti-neutrophil cytoplasmic
25 antibodies in the sample.

 25. The method as recited in claim 17, wherein the sample is one of human feces, whole blood, serum, plasma, human bodily fluid and human tissue.

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**FIG. 1**

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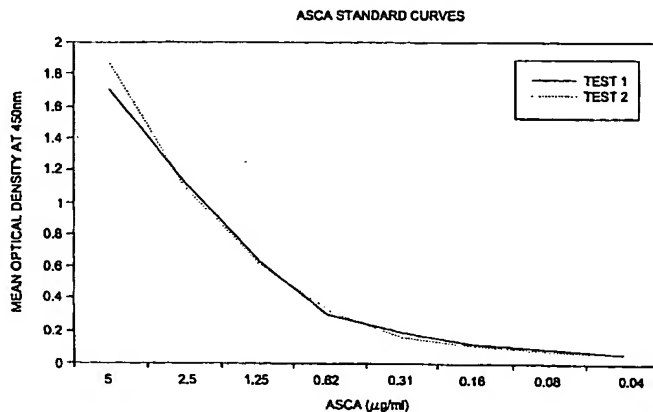
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(54) Title: METHOD AND APPARATUS FOR DISTINGUISHING CROHN'S DISEASE FROM ULCERATIVE COLITIS AND OTHER GASTROINTESTINAL DISEASES BY DETECTING THE PRESENCE OF FECAL ANTIBODIES TO SACCHAROMYCES CEREVISIAE



(57) Abstract: A method and apparatus for the differentiation of Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome, using the presence of fecal anti-Saccharomyces cerevisiae antibodies (ASCA) as a marker for Crohn's disease are provided. The apparatus includes an enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobins for the measurement of total endogenous ASCA in a human fecal sample. The method and apparatus may be used by healthcare providers to distinguish Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

A method and apparatus for the differentiation of Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome, using the presence of fecal anti-*Saccharomyces cerevisiae* antibodies (ASCA) as a marker for Crohn's disease are provided. The apparatus includes an enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobulins for the measurement of total endogenous ASCA in a human fecal sample. The method and apparatus may be used by healthcare providers to distinguish Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome.

It is estimated that at least one million Americans suffer from Inflammatory Bowel Disease (IBD). IBD is characterized by a chronic inflammatory response that results in histologic damage to the intestinal lining. IBD comprises two known clinical subtypes, Crohn's Disease (CD) and ulcerative colitis (UC). CD may involve the entire gastrointestinal tract and include inflammation extending into the transmural mucosa whereas UC affects solely the large bowel and includes inflammation of the innermost lining. Due to the differences between them, these two distinct diseases require a rapid differential diagnosis for optimal treatment. Conventional methods for differentiating between these clinical subtypes of IBD utilize multiple endoscopy examinations and histological analysis. These methods, however, do not permit quick differential diagnosis as each may require years for a diagnosis to be confirmed. As a result, methods are needed for the rapid differential diagnosis of CD and UC.

30 Serological methods for the differential diagnosis of CD and UC are known in the art. For example, it is known in the art to use the presence of serum anti-Saccharomyces cerevisiae antibodies (ASCA) to diagnose CD. See Main et al., Antibody to Saccharomyces cerevisiae (baker's yeast) in Crohn's

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disease, BMJ Vol. 297 (October 29, 1988); Broker et al., A Murine Monoclonal Antibody Directed Against a Yeast Cell Wall Glycoprotein Antigen of the Yeast Genus *Saccharomyces*, FEMS Microbiology Letters 118 (1994), 297-304. It is further known in the art to use the presence of serum ASCA to diagnose clinical subtypes of UC and CD in patients presenting with established diagnoses. For example, U.S. Patent No. 5,968,741 discloses utilizing the presence of serum ASCA to diagnose a medically resistant clinical subtype of UC in patients presenting with an established diagnosis of UC. Similarly, U.S. Patent No. 5,932,429 discloses utilizing the presence of serum ASCA to diagnose a clinical subtype of CD in patients presenting with an established diagnosis of CD.

Each of the above-mentioned serological methods utilizing ASCA as a marker has a number of drawbacks. For instance, each method requires an invasive procedure such as a finger prick or the like to obtain a serum sample. Further, each method utilizes only serum antibodies that are not required to cross the intestinal wall and the serum antibodies may not be accurate indicator for the proper diagnosis.

SUMMARY OF THE INVENTION

A method for testing a fecal sample, the method comprising: obtaining a fecal sample from a person; and determining the amount of anti-*Saccharomyces cerevisiae* antibodies in the sample.

A method for testing a fecal sample, the method comprising: obtaining a fecal sample from a person; and determining the presence of anti-*Saccharomyces cerevisiae* antibodies in the sample, wherein the presence of fecal anti-*Saccharomyces cerevisiae* antibodies is used to aid in the differentiation of Crohn's disease from other gastrointestinal illnesses such as, ulcerative colitis and irritable bowel syndrome (IBS).

An assay for determining the concentration of endogenous anti-*Saccharomyces cerevisiae* antibodies, the assay comprising: obtaining a human fecal sample; diluting the fecal sample; contacting the sample with extract of *Saccharomyces cerevisiae* to create a treated sample; contacting the treated sample with enzyme-linked polyclonal antibodies to create a readable sample; determining the optical density of the readable sample at 450 nm; generating a

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purified anti-Saccharomyces cerevisiae antibodies standard curve; and comparing the optical density of the readable sample to the standard curve to determine the concentration of endogenous anti-Saccharomyces cerevisiae antibodies in the fecal sample.

5 A diagnostic assay for diagnosing Crohn's disease by determining the level of endogenous anti-Saccharomyces cerevisiae antibodies , the assay comprising: obtaining a human fecal sample; diluting the sample; contacting the sample extract Saccharomyces cerevisiae to create a treated sample; contacting the treated sample with enzyme-linked polyclonal antibodies to create a readable
10 sample; adding an enzyme substrate for color development and determining the optical density of the readable sample at 450 nm to determine whether the readable sample contains an elevated level of endogenous anti-Saccharomyces cerevisiae antibodies as compared to a reference value for healthy control subjects.

15 A kit for diagnosing Crohn's disease by testing a fecal sample from a person to be diagnosed, the kit comprising: one or more microassay plates, each the plate containing extract Saccharomyces cerevisiae; enzyme-linked polyclonal antibody to human anti-Saccharomyces cerevisiae antibodies ; and enzyme substrate for color development.

20 Additional aspects of invention, together with the advantages and novel features appurtenant thereto, will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned from the practice of the invention. The objects and advantages of the invention may be realized and
25 attained by means, instrumentalities and combinations particularly pointed out in the appended claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 is a graphical representation of a standard curve of purified anti-Saccharomyces cerevisiae antibodies.

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DETAILED DESCRIPTION OF THE INVENTION

A method and apparatus for the differentiation of Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome, using the presence of fecal anti-Saccharomyces cerevisiae antibodies (ASCA) as a marker for Crohn's disease are provided. The apparatus includes an enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobulins for the measurement of total endogenous ASCA in a human fecal sample. The method and apparatus may be used by healthcare providers to distinguish Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome. The particular embodiments described herein are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present embodiment of the invention pertains without departing from its scope.

The present embodiment of the invention provides immunoassays, including, but not limited to, enzyme-linked immunoassays (ELISAs), that utilize antibodies specific to human ASCA for the measurement of total endogenous ASCA in fecal samples, including feces and mucosal secretions. The assay of the present invention may include, but is not limited to, the following steps: 1) obtaining a fecal sample from a person to be diagnosed, 2) diluting the sample, 3) contacting the sample with extract of Saccharomyces cerevisiae to create a treated sample, and 4) contacting the treated sample with enzyme-linked polyclonal antibodies to create a readable sample. Further, the optical density of the readable sample at 450 nm may be determined. The optical density of the readable sample then may be compared to a standard curve generated using purified anti-Saccharomyces cerevisiae standard curve to determine the concentration of the endogenous anti-Saccharomyces cerevisiae antibodies in the fecal sample. The present embodiment of the invention further provides a kit usable in such immunoassays to aid physicians and other clinical personnel in diagnosing Crohn's disease.

It will be understood and appreciated by those of skill in the art that a immunoassay such as a lateral flow dipstick that utilizes both monoclonal

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and polyclonal antibodies to total endogenous ASCA also may be used to diagnose Crohn's disease. Such is contemplated to be within the scope hereof.

A limited number of cases of ulcerative colitis and IBS may test positive for ASCA. Therefore, it is possible that a diagnosis of Crohn's disease cannot be established solely on the basis of a positive result with the assay of the present embodiment of the invention. However, a positive result with the assay of the present embodiment of the invention will permit the substantial preclusion of a diagnosis of a other gastrointestinal illness, such as IBS or ulcerative colitis.

The immunoassay of the embodiment of the present embodiment of the invention may be used as an in vitro diagnostic aid for detecting elevated levels of ASCA as a detection marker for Crohn's disease. The immunoassay of the present embodiment of the invention provides a test that is easy to use, simple to read, and accurate for distinguishing Crohn's disease from IBS or ulcerative colitis.

The following examples are intended in all respects to be illustrative rather than restrictive.

Example 1

In this example using an ELISA method, a fecal sample was obtained and serially diluted 20 fold. 100 µl of the diluted sample was added to a test well of a microassay plate coated with extract of *Saccharomyces cerevisiae* and purified mannan. The sample then was incubated at 37°C to allow antibodies to *Saccharomyces cerevisiae* to bind to the extract of *Saccharomyces cerevisiae*. Following incubation, anti-human Ig polyclonal antibodies coupled to horseradish peroxidase enzyme (conjugate) were added to the test well and allowed to bind to captured ASCA. Unbound conjugate then was washed from the well and one component substrate (tetra-methyl-benzidine and hydrogen peroxide) was added for color development. Following the substrate incubation, 0.1M sulfuric acid was added to quench the reaction and the optical density (OD) was obtained spectrophotometrically at 450 nm using a single wavelength spectrophotometer.

The method described above was used in a clinical study to test a total of 86 IBD patients (55.8% males and 44.2% females). The approximate 1 to

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1 ratio of males to females was similar to the ratio observed in IBD patient populations. The IBS patient group ranged in age from 19 to 78 years and was 9% male and 91% female. This ratio of males to females (1:10) reflects the increased incidence for IBS in females as seen in patient populations. The

5 healthy control (HC) patient group ranged in age from 20 to 79 years old and was 33.3% male and 66.6% female. A summary of the patient population in the clinical study is shown in Table 1.

TABLE 1 Summary of patient population.

10

Summary of Clinical Histories (N=120)	Total Subjects
Total number of IBD patients	86
No. Males	48
No. Females	38
Total number of patients with Crohn's Disease	49
No. Males	26
No. Females	23
Total number of patients with ulcerative colitis	37
No. Males	22
No. Females	15
Total number of patients with irritable bowel syndrome	22
No. Males	2
No. Females	20
Total number of healthy controls	12
No. Males	4
No. Females	8

In the clinical study, there were 37 ulcerative colitis patients, 49 Crohn's disease patients, 22 irritable bowel patients, and 12 healthy controls. Fecal samples were collected from each enrolled subject and stored at -70°C until

15 tested. The optical densities for each sample were determined using the method described above. Results were reported as positive for fecal ASCA if an optical density of greater than or equal to 0.200 was observed. Results were reported as negative for fecal ASCA if an optical density of less than or equal to 0.199 was observed. Other clinical data, such as stool consistency, was also determined.

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Table 2, below, contains the clinical data and test results for healthy patients that participated in this clinical study. Table 3, below, contains the clinical data and test results for patients with ulcerative colitis patients that participated in this clinical study. Table 4, below, contains the clinical data and test results for patients with Crohn's disease that participated in this study. Table 5, below, contains the clinical data and test results for patients with irritable bowel syndrome that participated in this study.

TABLE 2 Clinical data and test results for healthy controls

Donor ID	Sex	Age Range	Previous or chronic GI illness	Stool Consistency	Optical Density	Fecal ASCA
HC1	F	40 - 49	NO	Solid	0.098	NEGATIVE
HC2	F	40 - 49	NO	Solid	0.089	NEGATIVE
HC3	M	70 - 79	NO	Solid	0.095	NEGATIVE
HC4	F	60 - 69	NO	Solid	0.085	NEGATIVE
HC5	M	70 - 79	NO	Solid	0.083	NEGATIVE
HC6	F	70 - 79	NO	Solid	0.076	NEGATIVE
HC7	F	50 - 59	NO	Solid	0.124	NEGATIVE
HC8	F	40 - 49	NO	Solid	0.095	NEGATIVE
HC9	F	50 - 49	NO	Solid	0.111	NEGATIVE
HC10	F	40 - 49	NO	Solid	0.111	NEGATIVE
HC11	M	50 - 60	NO	Solid	0.070	NEGATIVE
HC12	M	50 - 60	NO	Solid	0.054	NEGATIVE

TABLE 3 Clinical data and test results for ulcerative colitis patients

Patient ID	Sex	Age	Disease	Stool Consistency	Disease Activity	Optical Density	Fecal ASCA
UC1	F	46	UC	Liquid	ACTIVE	0.184	NEGATIVE
UC2	M	39	UC	Liquid	ACTIVE	0.378	POSITIVE
UC3	F	30	UC	Semi-Solid	ACTIVE	0.193	NEGATIVE
UC4	F	31	UC	Semi-Solid	INACTIVE	0.319	POSITIVE
UC5	F	30	UC	Semi-Solid	ACTIVE	0.114	NEGATIVE
UC6	M	61	UC	Semi-Solid	INACTIVE	0.115	NEGATIVE
UC7	F	68	UC	Liquid	INACTIVE	0.091	NEGATIVE
UC8	F	45	UC	Liquid	ACTIVE	0.356	POSITIVE
UC9	F	21	UC	Semi-Solid	ACTIVE	0.082	NEGATIVE
UC10	F	27	UC	Liquid	ACTIVE	0.161	NEGATIVE

UC11	F	24	UC	Solid	INACTIVE	0.104	NEGATIVE
UC12	F	74	UC	Semi-Solid	INACTIVE	0.091	NEGATIVE
UC13	M	69	UC	Semi-Solid	ACTIVE	0.070	NEGATIVE
UC14	M	19	UC	Solid	INACTIVE	0.088	NEGATIVE
UC15	M	62	UC	Solid	INACTIVE	0.054	NEGATIVE
UC16	F	70	UC	Solid	INACTIVE	0.056	NEGATIVE
UC17	M	23	UC	Liquid	ACTIVE	0.573	POSITIVE
UC18	F	52	UC	Solid	ACTIVE	0.073	NEGATIVE
UC19	M	60	UC	Solid	INACTIVE	0.062	NEGATIVE
UC20	F	52	UC	Liquid	ACTIVE	0.089	NEGATIVE
UC21	M	31	UC	Solid	INACTIVE	0.064	NEGATIVE
UC22	M	44	UC	Semi-Solid	INACTIVE	0.143	NEGATIVE
UC23	F	30	UC	Liquid	ACTIVE	0.110	NEGATIVE
UC24	M	48	UC	Semi-Solid	INACTIVE	0.096	NEGATIVE
UC25	F	37	UC	Liquid	ACTIVE	0.282	POSITIVE
UC26	F	32	UC	Solid	ACTIVE	0.107	NEGATIVE
UC27	F	46	UC	Liquid	ACTIVE	0.199	NEGATIVE
UC28	M	49	UC	Semi-Solid	INACTIVE	0.161	NEGATIVE
UC29	F	42	UC	Solid	INACTIVE	0.080	NEGATIVE
UC30	F	41	UC	Semi-Solid	INACTIVE	0.087	NEGATIVE
UC31	F	43	UC	Solid	INACTIVE	0.070	NEGATIVE
UC32	M	30	UC	Solid	ACTIVE	0.103	NEGATIVE
UC33	F	43	UC	Solid	INACTIVE	0.092	NEGATIVE
UC34	F	33	UC	Semi-Solid	INACTIVE	0.075	NEGATIVE
UC35	M	58	UC	Semi-Solid	ACTIVE	0.121	NEGATIVE
UC36	F	32	UC	Semi-Solid	ACTIVE	0.083	NEGATIVE

TABLE 4 Clinical Data and test results for Crohn's disease patients.

Patient ID	Sex	Age	Disease	Stool Consistency	Disease Activity	Optical Density	FECAL ASCA
CD1	M	26	CD	Liquid	INACTIVE	1.900	POSITIVE
CD2	M	60	CD	Liquid	ACTIVE	2.849	POSITIVE
CD3	F	66	CD	Liquid	ACTIVE	0.282	POSITIVE
CD4	F	74	CD	Semi-Solid	INACTIVE	0.091	NEGATIVE
CD5	F	25	CD	Solid	INACTIVE	0.162	NEGATIVE
CD6	F	66	CD	Semi-Solid	INACTIVE	1.240	POSITIVE
CD7	M	39	CD	No Data	ACTIVE	1.150	POSITIVE
CD8	F	46	CD	Liquid	ACTIVE	0.160	NEGATIVE
CD9	F	46	CD	Semi-Solid	INACTIVE	0.074	NEGATIVE

CD10	F	56	CD	Solid	ACTIVE	0.406	POSITIVE
CD11	M	56	CD	Solid	ACTIVE	0.168	NEGATIVE
CD12	F	56	CD	Liquid	ACTIVE	0.732	POSITIVE
CD13	M	21	CD	Solid	ACTIVE	1.369	POSITIVE
CD14	M	52	CD	Semi-Solid	INACTIVE	0.136	NEGATIVE
CD15	M	63	CD	Solid	INACTIVE	0.134	NEGATIVE
CD16	M	34	CD	Solid	ACTIVE	0.076	NEGATIVE
CD17	F	45	CD	Semi-Solid	ACTIVE	0.160	NEGATIVE
CD18	M	67	CD	Semi-Solid	INACTIVE	0.059	NEGATIVE
CD19	F	46	CD	No Data	ACTIVE	0.839	POSITIVE
CD20	M	66	CD	Semi-Solid	INACTIVE	0.084	NEGATIVE
CD21	M	63	CD	Liquid	ACTIVE	0.780	POSITIVE
CD21	M	51	CD	Semi-Solid	ACTIVE	3.000	POSITIVE
CD22	M	34	CD	Semi-Solid	ACTIVE	1.447	POSITIVE
CD23	M	21	CD	Solid	ACTIVE	2.757	POSITIVE
CD24	F	78	CD	Semi-Solid	INACTIVE	0.092	NEGATIVE
CD25	F	27	CD	Semi-Solid	ACTIVE	0.979	POSITIVE
CD26	M	40	CD	Liquid	ACTIVE	0.373	POSITIVE
CD27	M	51	CD	Liquid	ACTIVE	0.978	POSITIVE
CD28	M	42	CD	Liquid	ACTIVE	0.089	NEGATIVE
CD29	F	31	CD	Solid	INACTIVE	0.075	NEGATIVE
CD30	F	59	CD	Solid	ACTIVE	0.088	NEGATIVE
CD31	M	35	CD	Semi-Solid	ACTIVE	1.487	POSITIVE
CD32	M	37	CD	Semi-Solid	INACTIVE	1.257	POSITIVE
CD33	F	77	CD	Solid	INACTIVE	0.093	NEGATIVE
CD34	F	40	CD	No Data	ACTIVE	1.762	POSITIVE
CD35	F	38	CD	Liquid	ACTIVE	0.098	NEGATIVE
CD36	M	51	CD	Liquid	ACTIVE	2.326	POSITIVE
CD37	M	38	CD	Semi-Solid	ACTIVE	0.091	NEGATIVE
CD38	M	37	CD	Liquid	ACTIVE	0.372	POSITIVE
CD39	M	59	CD	Semi-Solid	ACTIVE	0.224	POSITIVE
CD40	F	41	CD	Solid	ACTIVE	0.503	POSITIVE
CD41	M	41	CD	Solid	ACTIVE	0.117	NEGATIVE
CD42	M	48	CD	Liquid	ACTIVE	0.115	NEGATIVE
CD43	F	40	CD	Solid	INACTIVE	0.638	POSITIVE
CD44	F	72	CD	Solid	ACTIVE	0.087	NEGATIVE
CD45	F	32	CD	Liquid	INACTIVE	0.911	POSITIVE
CD46	F	24	CD	Liquid	ACTIVE	0.341	POSITIVE
CD47	M	23	CD	Solid	INACTIVE	0.088	NEGATIVE
CD48	F	34	CD	Liquid	ACTIVE	0.599	POSITIVE

TABLE 5 Clinical data and test results for irritable bowel syndrome patients

Patient ID	Sex	Age	Disease	Stool consistency	Disease Activity	Optical Density	Fecal ASCA
IBS1	F	56	IBS	Semi-Solid	ACTIVE	0.132	NEGATIVE
IBS2	F	48	IBS	Solid	ACTIVE	0.103	NEGATIVE
IBS3	F	30	IBS	Solid	ACTIVE	0.073	NEGATIVE
IBS4	F	31	IBS	Solid	ACTIVE	0.074	NEGATIVE
IBS5	F	72	IBS	Semi-Solid	ACTIVE	0.079	NEGATIVE
IBS6	F	47	IBS	Solid	ACTIVE	0.088	NEGATIVE
IBS7	F	19	IBS	Semi-Solid	ACTIVE	0.105	NEGATIVE
IBS8	F	58	IBS	Semi-Solid	ACTIVE	0.107	NEGATIVE
IBS9	F	40	IBS	Solid	ACTIVE	0.065	NEGATIVE
IBS10	F	33	IBS	Semi-Solid	ACTIVE	0.065	NEGATIVE
IBS11	F	78	IBS	Solid	ACTIVE	0.071	NEGATIVE
IBS12	F	74	IBS	Semi-Solid	ACTIVE	0.063	NEGATIVE
IBS13	F	50	IBS	Semi-Solid	ACTIVE	0.052	NEGATIVE
IBS14	F	39	IBS	Solid	ACTIVE	0.079	NEGATIVE
IBS15	F	54	IBS	Solid	ACTIVE	0.080	NEGATIVE
IBS16	M	49	IBS	Semi-Solid	ACTIVE	0.238	POSITIVE
IBS17	M	53	IBS	Solid	ACTIVE	0.123	NEGATIVE
IBS18	F	34	IBS	Solid	ACTIVE	0.091	NEGATIVE
IBS19	F	43	IBS	Solid	ACTIVE	0.075	NEGATIVE
IBS20	F	35	IBS	Solid	ACTIVE	0.075	NEGATIVE
IBS21	F	51	IBS	Semi-Solid	ACTIVE	0.081	NEGATIVE
IBS22	F	40	IBS	Solid	ACTIVE	0.083	NEGATIVE

5 There were a total of 49 patients with Crohn's disease and 37 with
ulcerative colitis. In the Crohn's disease group, a total of 55.1% patients were
positive for fecal ASCA. In the ulcerative colitis group, 13.5% were positive. Of
the 22 IBS patients, a single patient (4.6%) was positive for fecal ASCA. All 12
healthy controls were negative. A summary of positive results for fecal ASCA is
10 shown in Table 6.

TABLE 6 Summary of positive results for Crohn's disease, ulcerative colitis, active IBS, and healthy controls

Total Assessments N = 120	Total	Fecal ASCA Positive	Fecal ASCA Negative
Total IBD (Crohn's disease and ulcerative colitis)	86	37.2% (32)	62.8% (54)
Total Crohn's Disease	49	55.1% (27)	44.9% (22)
Total Ulcerative Colitis	37	13.5% (5)	86.5% (32)
Total Active IBS	22	4.6% (1)	96.4% (21)
Total Healthy Controls	12	0	100.0% (12)

5 When distinguishing Crohn's disease from ulcerative colitis, fecal ASCA exhibited a sensitivity of 55.1% and specificity of 86.5%. The predictive positive and negative values were 84.4% and 59.3%, respectively, and the correlation was 68.6% as shown in Table 7.

10 **TABLE 7 Statistical evaluation using the presence of fecal ASCA to distinguish Crohn's disease from ulcerative colitis**

N=86	Crohn's disease	Ulcerative colitis
Fecal ASCA positive	27	5
Fecal ASCA negative	22	32

Sensitivity	55.1%
Specificity	86.5%
Predictive Positive Value	84.4%
Predictive Negative Value	59.3%
Correlation	68.6%

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When distinguishing Crohn's disease from ulcerative colitis, irritable bowel syndrome and healthy controls, fecal ASCA exhibited a sensitivity of 55% and a specificity of 91.6%. The predictive positive and negative values were 82% and 75%, respectively, and the correlation was 77% as shown below in

5 Table 8.

TABLE 8 Statistical evaluation using fecal ASCA to distinguish Crohn's disease from ulcerative colitis, irritable bowel syndrome/healthy controls

N=120	Crohn's disease	UC/IBS/Healthy Controls
Fecal ASCA positive	27	6
Fecal ASCA negative	22	65

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Sensitivity	55.1%
Specificity	91.6%
Predictive Positive Value	81.8%
Predictive Negative Value	74.7%
Correlation	76.7%

The mean optical densities for each group were obtained and differences were tested for statistical significance using a two-tailed t-test giving a p-value result. Of the 33 patients that tested positive for fecal ASCA, there were 27 CD, 5 UC, and 1 IBS. Sensitivity, specificity and overall correlation were 55.1%, 91.5% and 76.7%, respectively. ASCA-positive CD showed a higher mean \pm SD A450 of 1.183 \pm 0.794 as compared to 0.382 \pm 0.113 for UC and the single A450 of 0.091 \pm 0.038 for IBS. There was a significant difference between CD and all other subject groups. A summary of the statistical analysis is listed in Table 9.

20

TABLE 9 Summary of the Mean and P values of Optical Densities for Fecal ASCA

Test Group	Mean Optical Density	Standard Deviation	Optical Density Range	P Value
CD	1.183	0.794	0.341-3.000	CD vs UC,IBS,HC P < 0.005
UC	0.382	0.113	0.382-0.113	CD vs UC P < 0.05

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IBS	0.091	0.038	0.052-0.238	CD vs IBS P < 0.005
HC	0.091	0.019	0.054-0.124	CD vs HC P < 0.005

Example 2

In this example, the sensitivity of the fecal ASCA test was determined using serial two fold dilutions of highly purified ASCA antibodies. For the analysis, standard curves were generated using the kit diluent. The test was consistently positive at a concentration of 0.62 µg/mL as determined by a cutoff absorbency value of ≥ 0.200 . Individual results are shown below in Table 10. The standard curves are shown in FIG. 1.

TABLE 10 Standard curves generated using purified ASCA antibodies

Purified ASCA Antibodies (µg/mL)	Test 1	Test 2	Mean	Std Dev
5.00	1.702	1.856	1.779	0.108
2.50	1.117	1.099	1.108	0.012
1.25	0.634	0.624	0.629	0.007
0.62	0.303	0.329	0.316	0.018
0.31	0.191	0.164	0.177	0.019
0.16	0.115	0.113	0.114	0.001
0.08	0.090	0.077	0.083	0.009
0.04	0.063	0.065	0.064	0.001

Example 3

In this example, tests were conducted to determine what type of immunoglobulins (antibodies) were present in a fecal sample and in serum. The immunoglobulin typing was done for human IgA, human IgAsec, human IgD, human IgM, and human IgG. The immunoglobulin typing was done on a fecal sample from 6 Crohn's disease patients and 2 ulcerative colitis and on a serum control sample using pre-absorbed Ig-type specific conjugates. The serum control sample was obtained from a patient with a confirmed allergy to *Saccharomyces cerevisiae*.

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Of the Crohn's disease patients, 5 patients exhibited a response to IgA and IgAsec, 4 patients exhibited a response to IgM and a single patient exhibited a response to IgG. Of the 2 ulcerative colitis patients, a single patient reacted with the Ig conjugate. The serum control only exhibited a response to individual immunoglobulins IgM and IgG. A response to IgA and IgAsec occurred the fecal samples but not in the control serum sample. A summary of results are shown in Table 11.

10 **TABLE 11 A Summary of Immunoglobulin Typing of ASCA in a Human Fecal sample and a Serum Control**

Patient Number	Disease	IgA Conjugate	IgA _{sec} Conjugate	IgD Conjugate	IgM Conjugate	IgG Conjugate	Ig Conjugate
1	Crohn's Disease	+	+	-	+	+	+
2	Crohn's Disease	+	+	-	+	-	+
3	Crohn's Disease	-	-	-	-	-	-
4	Crohn's Disease	+	+	NO DATA	+	-	+
5	Crohn's Disease	+	+	NO DATA	-	-	+
6	Crohn's Disease	+	+	NO DATA	+	-	+
7	Ulcerative Colitis	-	-	-	-	-	-
8	Ulcerative Colitis	-	-	-	-	-	+
Serum Control	Yeast Allergy	-	-	-	+	+	+

- 15 -

In summary, the present embodiment of the invention provides a method and apparatus for the differentiation of Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome, using the presence of fecal anti-Saccharomyces cerevisiae antibodies (ASCA) as
5 a marker for Crohn's disease. The apparatus includes an enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobins for the measurement of total endogenous ASCA in a human fecal sample. The method and apparatus may be used by healthcare providers to distinguish Crohn's disease from other gastrointestinal illnesses, such as
10 ulcerative colitis and irritable bowel syndrome. The present embodiment of the invention has been described in relation to particular embodiments which are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present embodiment of the invention pertains without departing from its scope.

15 From the foregoing, it will be seen that this embodiment of the invention is one well adapted to attain all the ends and objects hereinabove set forth together with other advantages which are obvious and which are inherent to the method.

It will be understood that certain features and subcombinations are
20 of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims.

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CLAIMS

Having thus described the invention, what is claimed is:

1. A method for testing a fecal sample, the method comprising:
obtaining a fecal sample from a person; and determining the amount of anti-
5 *Saccharomyces cerevisiae* antibodies in the sample.
2. The method of claim 1, further comprising: determining
whether the sample contains an elevated level of endogenous anti-*Saccharomyces*
cerevisiae antibodies.
3. The method of claim 2, wherein if the sample does contain an
10 elevated level of anti-*Saccharomyces cerevisiae* antibodies, a diagnosis of
Crohn's disease may be substantially concluded.
4. The method of claim 1, wherein the amount of fecal anti-
Saccharomyces cerevisiae antibodies is used to aid in the differentiation of
Crohn's disease from ulcerative colitis.
- 15 5. The method of claim 1, wherein the amount of fecal anti-
Saccharomyces cerevisiae antibodies is used to aid in the differentiation of
Crohn's disease from other gastrointestinal illnesses.
6. The method of claim 5, wherein the other gastrointestinal
illness is irritable bowel syndrome.
- 20 7. The method as recited in claim 1, wherein the endogenous
anti-*Saccharomyces cerevisiae* antibodies comprise the total anti-*Saccharomyces*
cerevisiae antibodies.
8. The method as recited in claim 1, wherein the endogenous
antibodies are secretory IgA.
- 25 9. The method as recited in claim 1, further comprising diluting
the fecal sample.
10. The method as recited in claim 9, wherein the step of diluting
the fecal sample comprises diluting the sample to a 1:20 dilution factor.

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11. The method as recited in claim 9, wherein determining the amount of anti-*Saccharomyces cerevisiae* antibodies in the sample further includes contacting the sample with extract of *Saccharomyces cerevisiae* and to create a treated sample.

5 12. The method as recited in claim 11, wherein the step of determining the amount of endogenous anti-*Saccharomyces cerevisiae* antibodies further includes contacting the treated sample with enzyme-linked polyclonal antibodies to create a readable sample.

10 13. The method as recited in claim 12, wherein the step of determining the amount of anti-*Saccharomyces cerevisiae* antibodies further includes determining an optical density of the readable sample at 450 nm, wherein the optical density corresponds to a level of endogenous anti-*Saccharomyces cerevisiae* antibodies in the sample.

15 14. The method as recited in claim 13, wherein if the optical density of the readable sample is greater than or equal to 0.200, the fecal sample contains an elevated level of endogenous anti-*Saccharomyces cerevisiae* antibodies.

15. The method of claim 1, wherein the fecal sample includes human feces and mucosal secretions.

20 16. An assay for determining the concentration of endogenous anti-*Saccharomyces cerevisiae* antibodies, the assay comprising: obtaining a human fecal sample; diluting the fecal sample; contacting the sample with extract of *Saccharomyces cerevisiae* to create a treated sample; contacting the treated sample with enzyme-linked polyclonal antibodies to create a readable sample;
25 determining the optical density of the readable sample at 450 nm; generating a purified anti-*Saccharomyces cerevisiae* antibodies standard curve; and comparing the optical density of the readable sample to the standard curve to determine the concentration of endogenous anti-*Saccharomyces cerevisiae* antibodies in the fecal sample.

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17. A diagnostic assay for diagnosing Crohn's disease by determining the level of endogenous anti-*Saccharomyces cerevisiae* antibodies, the assay comprising: obtaining a human fecal sample; diluting the sample; contacting the sample extract *Saccharomyces cerevisiae* to create a treated sample; contacting the treated sample with enzyme-linked polyclonal antibodies to create a readable sample; adding an enzyme substrate for color development; and determining the optical density of the readable sample at 450 nm to determine whether the readable sample contains an elevated level of endogenous anti-*Saccharomyces cerevisiae* antibodies as compared to a reference value for healthy control subjects.

18. The diagnostic assay as recited in claim 17, wherein if the readable sample contains an elevated level of endogenous anti-*Saccharomyces cerevisiae* antibodies, a diagnosis of Crohn's disease is substantially concluded.

19. The diagnostic assay as recited in claim 18, wherein if the optical density of the readable sample is greater than or equal to 0.200, the fecal sample contains endogenous anti-*Saccharomyces cerevisiae* antibodies.

20. The diagnostic assay as recited in claim 17, wherein the assay comprises an enzyme-linked immunoassay.

21. A kit for diagnosing Crohn's disease by testing a fecal sample from a person to be diagnosed, the kit comprising: one or more microassay plates, each the plate containing extract *Saccharomyces cerevisiae*; enzyme-linked polyclonal antibody to human anti-*Saccharomyces cerevisiae* antibodies; and enzyme substrate for color development.

22. The kit as recited in claim 21, further comprising purified human anti-*Saccharomyces cerevisiae* antibodies as a positive control.

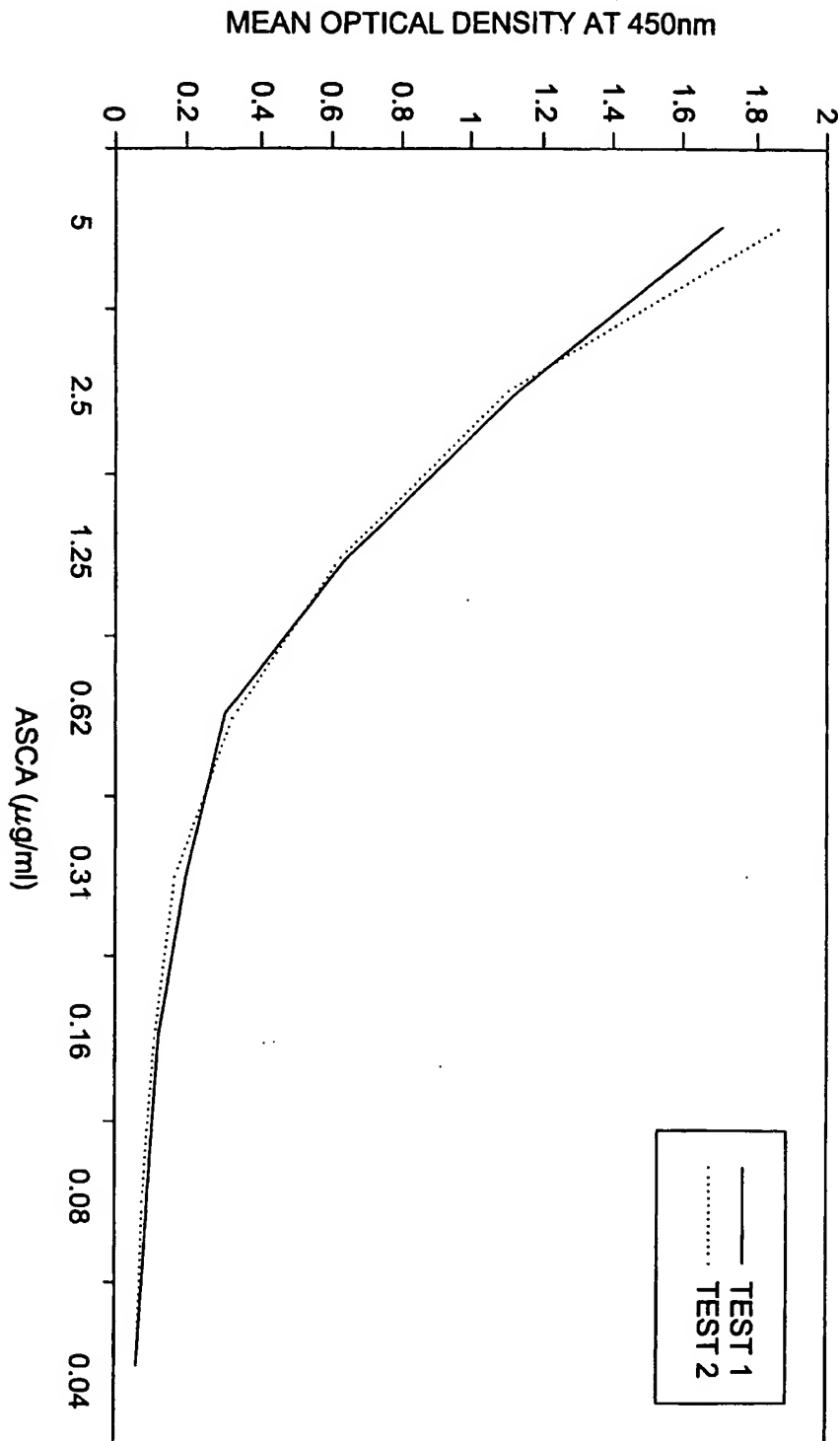
23. The kit as recited in claim 21, further comprising a stop solution for quenching the reaction.

24. The kit as recited in claim 22, further comprising a stop solution for quenching the reaction.

30

FIG. 1

ASCA STANDARD CURVES



DOCUMENT-IDENTIFIER: US 20040241823 A1

TITLE: Method and kit for the diagnosis of ulcerative colitis

Summary of Invention Paragraph:

[0008] For example WO 03/036262 describes a method and apparatus for the differentiation of Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome, using the presence of faecal anti-Saccharomyces cerevisiae antibodies (ASCA) as a marker for Crohn's disease are provided. The apparatus includes an enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobins for the measurement of total endogenous ASCA in a human faecal sample. The method and apparatus may be used by healthcare providers to distinguish Crohn's disease from other gastrointestinal illnesses, such as ulcerative colitis and irritable bowel syndrome.

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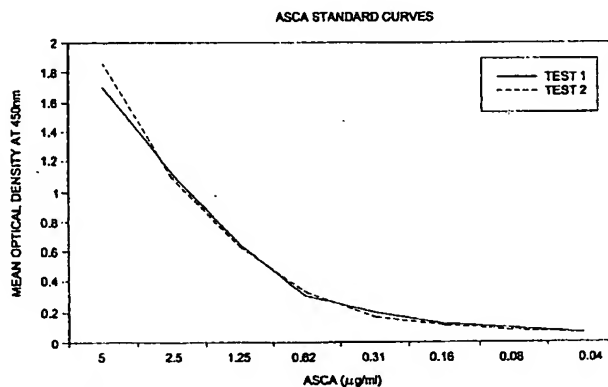
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[Continued on next page]

(54) Title: INFLAMMATORY BOWEL DISEASE AND IRRITABLE BOWEL SYNDROME IBD-FIRST CHEK DIAGNOSTIC PANEL



(57) Abstract: A method for the differentiation of inflammatory bowel disease (IBD) FROM IRRITABLE BOWEL DISEASE (IBS) followed by distinguishing ulcerative colitis and Crohn's disease from other gastrointestinal illnesses. This highly differential method first uses the presence of elevated lactoferrin as a marker of intestinal inflammation to differentiate IBD from IBS. Patients suspected of IBD are then analyzed for fecal anti-*Saccharomyces cerevisiae* antibodies (ASCA) AS AN INDICATOR OF Crohn's disease and fecal anti-neutrophil cytoplasmic antibodies (ANCA) as an indicator of ulcerative colitis. IBD patients are further monitored for intestinal inflammation using fecal lactoferrin to evaluate the effectiveness of medical therapy and to predict relapse. The apparatus comprises either a qualitative enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to human immunoglobins for the measurement of total endogenous lactoferrin, ASCA and ANCA in human feces. The method and apparatus can be used by healthcare providers to identify IBD and distinguish ulcerative colitis from Crohn's disease.

WO 2004/037073 A2



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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INFLAMMATORY BOWEL DISEASE AND IRRITABLE BOWEL
SYNDROME IBD-FIRST CHEK DIAGNOSTIC PANEL

TECHNICAL FIELD

A method for the differentiation of inflammatory bowel disease
5 (IBD) from irritable bowel disease (IBS) followed by distinguishing ulcerative
colitis from Crohn's disease and other gastrointestinal illnesses. This highly
differential method first uses the presence of elevated fecal lactoferrin as a
marker of intestinal inflammation to differentiate IBD from IBS. Patients
suspected of IBD are then analyzed for anti-*Saccharomyces cerevisiae* antibodies
10 (ASCA) as an indicator of Crohn's disease and fecal anti-neutrophil cytoplasmic
antibodies (ANCA) as an indicator of ulcerative colitis. IBD patients are further
monitored for intestinal inflammation using lactoferrin to evaluate the
effectiveness of medical therapy and predict relapse. The apparatus comprises
either a qualitative enzyme-linked immunoassay or other immunoassay that
15 utilizes antibodies for the measurement of total endogenous lactoferrin, ASCA
and ANCA in human feces. The method and apparatus can be used by healthcare
providers to identify IBD and distinguish ulcerative colitis from Crohn's disease.

BACKGROUND OF THE INVENTION

An estimated 1 million Americans suffer from chronic
20 inflammatory bowel disease (IBD) and 20 million Americans suffer from irritable
bowel syndrome (IBS). IBD, comprised of both Crohn's Disease (CD) and
ulcerative colitis (UC), is characterized by a chronic inflammatory response that
results in histologic damage to the intestinal lining. Both CD and UC exhibit
large numbers of leukocytes that migrate to the mucosa and into the intestinal
25 lumen. Both diseases oscillate between active (i.e., presence of intestinal
inflammation) and inactive (i.e., minimal to no intestinal inflammation) stages of
disease activity. Active IBD can include symptoms such as bloody diarrhea,
abdominal pain, and fever. The inactive stage has minimal to no intestinal
inflammation and lacks severe gastrointestinal illness.

30 Patients who have active IBD but who exhibit mild signs and
symptoms may be difficult to distinguish from patients with active IBS, an
intestinal disorder of motility and the intestinal nervous system. Unlike IBD, IBS

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does not involve intestinal inflammation. In persons with IBS, the intestine appears normal upon endoscopic examination and leukocytes are not present in the mucosa or in fecal specimens. Symptoms can mimic those of IBD and include bloating, diarrhea, constipation, and severe and often debilitating abdominal pain. It is estimated that at least 20 million Americans suffer from IBS.

The similarity in symptoms between IBS and IBD renders rapid diagnosis difficult. However, given the potential severity of untreated IBD, differential diagnosis is crucial. The diagnosis of gastrointestinal illnesses, in general, is aided by diagnostic tests such as enzyme-linked immunosorbant assays (ELISAs), latex agglutination and lateral flow immunoassay. These tests are rapid and inexpensive methods for detecting markers in feces for enteric pathogens and inflammation. One marker of particular interest that has been found to be most specific for leukocytes in fecal specimens is lactoferrin. Human lactoferrin is an 80 kilodalton glycoprotein. This iron-binding protein is secreted by most mucosal membranes. It is a major component of the secondary granules found in polymorphonuclear neutrophils (PMNs), a primary component of the acute inflammatory response. Other hematopoietic cells such as monocytes and lymphocytes, do not contain lactoferrin, whereas various bodily secretions contain levels in the mg/mL range. During the process of inflammation, PMNs infiltrate the mucosa lining of the small and large intestine. This increase in the number of activated tissue leukocytes and exudation of plasma from ulcerated mucosa results in an increase in the level of lactoferrin found in feces. The protein is resistant to proteolysis and, as such, it provides a useful non-invasive fecal marker of intestinal inflammation.

Human lactoferrin has been used as a marker for fecal leukocytes in a number of applications. For instance, fecal lactoferrin has been used as a marker for leukocytes to distinguish noninflammatory diarrhea from inflammatory diarrhea, as disclosed in U.S. Patent No. 5,124,252. Noninflammatory diarrhea caused by agents such as rotavirus, Norwalk-like agents and cholera, typically causes minimal to no intestinal damage and patients respond readily to oral rehydration. Inflammatory diarrheas include those caused by enteric pathogens such as *Clostridium difficile*, *Shigella* species, *Salmonella*

species, *Campylobacter jejuni* and *Entamoeba histolytica* and those that have no clearly defined infectious agent such as CD and UC. U.S. Patent No. 5,124,252 discloses an *in vitro* test for fecal leukocytes that aids in distinguishing inflammatory from noninflammatory diarrhea. The '252 patent discloses testing
5 fecal samples suspected of containing leukocytes with an assay that utilizes an antibody for lactoferrin to determine the presence of leukocytes in the fecal sample.

Human lactoferrin also has been used as a marker for diagnosis of inflammatory gastrointestinal disorders, colon polyp and colorectal cancer as
10 disclosed in U.S. Patent No. 5,552,292. However, neither the method of the '252 patent nor that of the '292 patent disclose utility in distinguishing IBS from IBD. The samples tested by the assay of the '252 patent are samples suspected of containing leukocytes. This suspicion is owed to the patient presenting with diarrhea. However, 25-50% of persons having IBD do not present with diarrhea
15 and, thus, the '252 patent does not relate to diagnosing etiology in such patients. As for the '292 patent, the disclosed method utilizes a 1:100 sample dilution which does not allow for accurate quantitation of lactoferrin levels. Further, the '292 patent discloses using partial forms of molecules for testing and not total endogenous lactoferrin, again affecting the accuracy of the quantitation. The
20 method of the '292 patent also does not relate to utilizing lactoferrin levels to distinguish IBD from IBS. The population tested in the '292 patent, while including persons with UC and CD, did not include persons having IBS.

IBD is comprised of both Crohn's disease and ulcerative colitis. These two distinct diseases require a rapid differential diagnosis for optimal
25 treatment. Crohn's disease may involve the entire gastrointestinal tract and include inflammation extending into the transmural mucosa, whereas ulcerative colitis affects solely the large bowel and includes inflammation of the innermost lining. Conventional methods to differentiate between Crohn's disease and ulcerative colitis utilizing multiple endoscopy examinations and histological
30 analysis may take years to confirm a diagnosis.

U.S. Patent No. 6,218,120 discloses a method of determining the presence of serum ANCA as a marker to diagnose IBD. However, it does not

disclose a method for diagnosing ulcerative colitis in a patient diagnosed with IBD.

Serological methods for the differential diagnosis of CD and UC also are known in the art. For example, it is known to use the presence of serum anti-*Saccharomyces cerevisiae* antibodies (ASCA) to diagnose CD. See Main et al., Antibody to *Saccharomyces cerevisiae* (baker's yeast) in Crohn's disease, BMJ Vol. 297 (October 29, 1988); Broker et al., A Murine Monoclonal Antibody Directed Against a Yeast Cell Wall Glycoprotein Antigen of the Yeast Genus *Saccharomyces*, FEMS Microbiology Letters 118 (1994), 297-304. It is further known in the art to use the presence of serum ASCA to diagnose clinical subtypes of UC and CD in patients presenting with established diagnoses. For example, U.S. Patent No. 5,968,741 discloses utilizing the presence of serum ASCA to diagnose a medically resistant clinical subtype of UC in patients presenting with an established diagnosis of UC. Similarly, U.S. Patent No. 5,932,429 discloses utilizing the presence of serum ASCA to diagnose a clinical subtype of CD in patients presenting with an established diagnosis of CD.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 is a graphical representation of a standard curve of purified anti-*Saccharomyces cerevisiae* antibodies in accordance with an embodiment of the present invention; and

FIG. 2 is graphical representation of a standard curve of anti-neutrophil cytoplasmic antibodies in accordance with an embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates a lactoferrin immunoassay used to determine the presence of elevated lactoferrin as an indicator of intestinal inflammation thus aiding in the differentiation of IBD from IBS, and ANCA and ASCA immunoassays to differentiate between ulcerative colitis and Crohn's disease. The test results may be used to determine appropriate treatment for ulcerative colitis and Crohn's disease patients. Qualitative immunoassays such as enzyme-linked immunoassays and lateral flow dipsticks that utilize monoclonal and polyclonal antibodies to human ANCA and ASCA may be used distinguish

between ulcerative colitis and Crohn's disease. Bodily secretions, as used herein may include, but are not limited to, feces and mucosal secretions, whole blood, serum, plasma, saliva or other bodily fluid or tissue.

In the qualitative assay, the bodily secretions are diluted and added
5 to a well containing the immobilized antibodies to lactoferrin or antigens of *Saccharomyces cerevisiae* or neutrophil cytoplasmic antigens. If endogenous lactoferrin or ASCA or ANCA is present, it will bind to the well containing immobilized antibodies or antigens during an incubation step. Following the incubation, antibodies to human lactoferrin or polyvalent antibodies to human
10 immunoglobulin coupled to horseradish peroxidase enzyme (conjugate) is added and allowed to bind to captured lactoferrin or ANCA or ASCA. Unbound conjugate is washed from the well and one component substrate (tetra-methylbenzidine and hydrogen peroxide) is added for color development. Following the substrate incubation, the reaction is stopped by acidification and the optical
15 density (OD) is determined spectrophotometrically at 450 nm.

The particular embodiments described herein are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its scope.

20

Example 1
Lactoferrin Qualitative Assay

a. Establishment of Optimal Sample Dilution Factor and Optical Density

The assay of the present invention was designed and developed to
25 detect levels of fecal lactoferrin at a lower level detectable by predicate devices, specifically the LEUKO-TEST®. The lower limit of detection of the LEUKO-TEST® is 256 ng/mL with purified human lactoferrin. In the LEUKO-TEST®, a specimen dilution of 1:50 and a minimum limit of detection of 256 ng/mL provides a lower limit of detection in fecal specimens of approximately 12
30 µg/mL. A specimen dilution of 1:400 and a minimum detection limit for the assay of the present invention of 32 ng/mL also provides a lower limit of detection in fecal specimens of approximately 12 µg/mL. Accordingly, a 1:400 specimen dilution was chosen for the assay of the present invention. Similarly,

an optical density of 0.200 OD₄₅₀ for the assay was chosen. (As used herein, OD₄₅₀ indicates an optical density obtained spectrophotometrically at 450 nm on a single wavelength spectrophotometer.)

It will be understood and appreciated by those of skill in the art that the preferred dilution factor and optical densities have been determined based upon reagents currently available and deemed to be optimal. However, reagents other than those now desired may become improved and desirable over time. Variations in reagents may produce preferable/optimal dilution factors and/or optical densities other than those determined herein. Such variations are contemplated to be within the scope of the present invention. The key to determining optimal values is based upon sensitivity as more fully described below.

To verify that the 1:400 specimen dilution provides the most desirable sensitivity with the current reagents, 121 fecal specimens were analyzed comparing a 1:400 dilution to a 1:800 dilution. (Sensitivity is calculated herein by dividing the number of samples taken from subjects with IBD which produce a positive result in the assay by the number of samples taken from subjects with IBD.) Test results additionally were evaluated comparing OD₄₅₀ values of 0.200 to OD₄₅₀ values of 0.300. Results were compared with microscopy for fecal leukocytes and with the LEUKO-TEST®. The results are summarized in Tables 1-8 below.

Table 1: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:400 dilution and an OD₄₅₀ of 0.200

ELISA positive	32	42
ELISA negative	2	45

25

Relative Sensitivity	94.0%
Relative Specificity	52.0%
Correlation	64.0%

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Table 2: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:400 dilution and an OD₄₅₀ of 0.300

ELISA	Microscopy	Agreement
ELISA positive	31	31
ELISA negative	3	56

Relative Sensitivity	91.0%
Relative Specificity	64.0%
Correlation	72.0%

5

Table 3: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:800 dilution and an OD₄₅₀ of 0.200

ELISA	Microscopy	Agreement
ELISA positive	30	31
ELISA negative	4	56

10

Relative Sensitivity	88.0%
Relative Specificity	64.0%
Correlation	77.0%

Table 4: Comparison of the ELISA with microscopy for fecal leukocytes using a 1:800 dilution and an OD₄₅₀ of 0.300

ELISA	Microscopy	Agreement
ELISA positive	26	24
ELISA negative	8	63

15

Relative Sensitivity	77.0%
Relative Specificity	72.0%
Correlation	74.0%

Table 5: Comparison of the ELISA with the LEUKO-TEST[®] using a 1:400 dilution and an OD₄₅₀ of 0.200

ELISA	LEUKO-TEST [®]	ELISA
ELISA positive	43	31
ELISA negative	5	42

Relative Sensitivity	89.6%
Relative Specificity	57.5%
Correlation	70.2%

5

Table 6: Comparison of the ELISA with the LEUKO-TEST[®] using a 1:400 dilution and an OD₄₅₀ of 0.300

ELISA	LEUKO-TEST [®]	ELISA
ELISA positive	41	21
ELISA negative	7	52

Relative Sensitivity	85.0%
Relative Specificity	71.2%
Correlation	77.0%

10

Table 7: Comparison of the ELISA with the LEUKO-TEST[®] using a 1:800 dilution and an OD₄₅₀ of 0.200

ELISA	LEUKO-TEST [®]	ELISA
ELISA positive	39	22
ELISA negative	9	51

Relative Sensitivity	81.3%
Relative Specificity	69.9%
Correlation	74.4%

15

**Table 8: Comparison of the ELISA with the LEUKO-TEST®
using a 1:800 dilution and an OD₄₅₀ of 0.300**

ELISA positive	34	16
ELISA negative	14	57

Relative Sensitivity	70.8%
Relative Specificity	78.1%
Correlation	75.2%

5

In summary, a fecal specimen dilution of 1:400 and an assay OD₄₅₀ of 0.200 showed the highest level of sensitivity with the current reagents. Accordingly, these conditions were determined to be optimal for the assay of the present invention. Normal fecal specimens contain low levels of lactoferrin and the 1:400 dilutions have been determined to be optimal in detecting an increase in lactoferrin over background levels. The use of dilutions lower than 1:400 may result in positive test results due to the presence of normal lactoferrin levels.

10

b. Collection of Specimens and Preparation of Dilutions

Standard collection and handling procedures typically used for fecal specimens for culture may be used in collecting samples for the assay of the present invention. In the preferred embodiment, fecal specimens are to be tested within twenty-four hours of collection. However, if the assay is not to be performed within forty-eight hours of collection, it is preferred that the specimens be stored at -20°C or lower. Additionally, it is preferred that collected specimens be transported and diluted in the Diluent as soon as possible after collection and, once diluted, that the specimens be stored at between about 2°C and about 8°C. It is preferred that the specimens be mixed (i.e., using a vortex mixer) thoroughly prior to performing the assay of the present invention. This includes complete mixing of the specimen prior to transfer to the Diluent, as more fully described below, as well as complete mixing of the diluted specimen prior to performing the assay.

15

20

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- 10 -

The following method was used to prepare a diluted specimen from a liquid fecal specimen. Two plastic tubes were set up for each specimen to be tested. For each specimen, 950 μ L of 1X Diluent (prepared as more fully described below) subsequently was added to each of the two tubes. Using a transfer pipette, one drop (i.e., approximately 50 μ L) of liquid fecal specimen was added to one of the tubes and thoroughly mixed using a vortex mixer. Subsequently, one drop of the diluted specimen was transferred into the second tube containing 950 μ L of 1X Diluent (prepared as more fully described below). The result was a 1:400 dilution of the specimen in the second tube. Thus, only the second tube was used for the remainder of the test procedure.

The following method was used to prepare a diluted specimen from a formed or solid fecal specimen. Two plastic tubes were set up for each specimen to be tested. For each specimen, 1.9 mL of 1X Diluent (prepared as more fully described below) was added to only one of the two tubes. Subsequently, 0.10 g of fecal specimen were added to this tube (1:10) and thoroughly mixed using a vortex mixer. Next, 950 μ L of the 1X Diluent (prepared as more fully described below) was added to the second tube and one drop (i.e., approximately 50 μ L) of the previously diluted specimen is transferred into the second tube. The result was a 1:400 dilution of the specimen in the second tube. Thus, only the second tube was used for the remainder of the test procedure.

The specimen in the second tube prepared according to either of the above procedures was mixed in a vortex mixer for approximately ten seconds and subsequently stored at between about 2°C and about 8°C until the remainder of the test procedure was performed. Prior to transferring the diluted specimen into a microtiter well according to the test procedure, as more fully described below, the specimen was thoroughly mixed in the vortex mixer once again. This procedure sought to ensure thorough mixing of the specimen.

c. Necessary Test Reagents and Preparation Thereof

A number of reagents were necessary to carry out the preferred embodiment of the qualitative assay of the present invention. These reagents included 10X Diluent, 1X Diluent, Conjugate, Substrate, Positive Control, Wash Buffer Solution and Stop Solution. The 10X Diluent was a 10X concentrate of

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buffered protein solution containing 0.2% thimerosal as a preservative. The Diluent was supplied as a 10X concentrate. Therefore, to prepare the 1X Diluent necessary for the assay of the present invention, a total volume of 400 mL was diluted by adding 40 mL of the 10X concentrate to 360 mL of deionized water.

5 Any unused 1X Diluent was stored at between about 2°C and about 8°C.

The Conjugate used with the assay of the present invention preferably comprises rabbit polyclonal antibody specific for human lactoferrin conjugated to horseradish peroxidase and in a buffered protein solution containing 0.02% thimerosal as a preservative. The Substrate used with the assay
10 of the present invention preferably comprises a solution containing tetra-methyl-benzidine substrate and peroxidase. The Positive Control used with the assay of the present invention preferably comprises human lactoferrin in a buffered protein solution containing 0.02% thimerosal as a preservative. The Stop
15 Solution used with the assay of the present invention preferably comprises 0.6 N sulfuric acid.

The Wash Buffer Solution used with the assay of the present invention was supplied as a 20X concentrate containing phosphate buffered saline, detergent and 0.2% thimerosal as a preservative. To prepare the 1X Wash
20 Solution necessary for the assay of the present invention, a total volume of one liter of concentrate was diluted by adding 50 mL of the concentrate to 950 mL of deionized water. Any unused 1X Wash Solution was stored at between about 2°C and about 8°C.

Microassay plates containing twelve strips and eight wells per strip are preferred for the assay of the present invention. Each specimen and each
25 control requires a single coated well. To prepare the plates, each strip was coated with purified polyclonal antibody specific for lactoferrin. Microassay plates were stored with desiccant.

All reagents were stored at room temperature prior to use in the assay of the present invention.

30 The present invention includes a kit designed and prepared for carrying out the quantitative assay. In the preferred embodiment, the kit contains 40 mL 10X Diluent, 7 mL Conjugate, 14 mL Substrate, 3.5 mL Positive Control, 50 mL Wash Buffer Solution, 7 mL Stop Solution and one microassay plate

stored with desiccant. The assay of the present invention utilizes antibodies to human lactoferrin. The microassay plate supplied with the kit contains immobilized polyclonal antibody against lactoferrin. The detecting antibody consists of polyclonal antibody conjugated to horseradish peroxidase.

5 **d. Test Procedure**

To perform the qualitative assay of the present invention, initially the number of wells needed was determined. Each specimen or control required one well and, therefore, the number of wells was determined accordingly. Next, one drop (i.e., about 50 μ L) of Positive Control was added to a single well designated the Positive Control Well and one drop (i.e., about 50 μ L) of 1X Diluent was added to a single well designated the Negative Control Well. Subsequently, two drops (i.e., about 100 μ L) of 1:400 diluted specimen (prepared according to the above procedure) was added to a third well and all wells were incubated at about 37°C (\pm 2°C) for approximately thirty minutes. After incubation, the contents of the assay wells was discarded into a discard pan.

Next, each well was washed using 1X Wash Solution (prepared as described above) and placed in a squirt bottle with a fine-tipped nozzle. In this manner, the 1X Wash Solution was directed into the bottom of each of the wells with some force. Each well was filled with the 1X Wash Solution and the contents thereof subsequently discarded into a discard pan. The microassay plate was then inverted and slapped on a dry paper towel. This wash procedure was performed a minimum of four times using a dry paper towel each time. If any particulate matter was observed in the wells, the washing procedure was continued until all the matter was removed.

Subsequently, one drop (i.e., about 50 μ L) of Conjugate was added to each well and the wells were incubated at about 37°C (\pm 2°C) for approximately thirty minutes. After incubation, the contents of the assay wells were discarded into a discard pan and the washing procedure was repeated. Next, two drops (i.e., about 100 μ L) of Substrate were added to each well and the wells were gently tapped to mix the contents. The wells were then incubated at room temperature for approximately fifteen minutes. The wells were gently tapped a couple of times during the incubation period.

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Next, one drop (i.e., 50 μ L) of Stop Solution was added to each well and the wells were gently tapped. The wells were allowed to sit at room temperature for about two minutes before reading. The addition of Stop Solution converted the blue color to a yellow color which could then be quantified by measuring the optical density at 450 nm on a microplate ELISA reader. The instrument was blanked against the negative control and the underside of each well was wiped before measuring the optical density. Optical densities (OD_{450} and $OD_{450/620}$) were recorded for the Positive Control Well, the Negative Control Well and each specimen tested. (" $OD_{450/620}$ " as used herein indicates an optical density obtained spectrophotometrically at 450/620 nm on a dual wavelength spectrophotometer.) Readings of duplicate wells were averaged before the results were interpreted.

The specified test procedure represents the preferred embodiment as optimal results are obtained by following the procedure specified because the reagents, concentrations, incubation conditions, and processing specifications have been optimized for sensitivity and specificity. Accordingly, alterations of the specified procedure and/or of the indicated test conditions may affect the sensitivity and specificity of the test.

e. Quality Control

The positive and negative control must meet certain criteria for the test to be valid. First of all, the Positive Control Well must be a visible yellow color and, when read on a spectrophotometer, it must have an OD_{450} and $OD_{450/620} > 0.500$. The Negative Control Well must have an $OD_{450} < 0.200$ or an $OD_{450/620} < 0.160$. To ensure that carryover has not occurred, testing should be repeated if a sample gives a weak positive result (i.e., < 0.400) and is adjacent to a strong positive well.

f. Interpretation of Results

Optical densities were measured at 450 nm on a single wavelength spectrophotometer and at 450/620 nm on a dual wavelength spectrophotometer. On a single wavelength spectrophotometer, an OD_{450} of less than 0.200 indicated a negative result and an OD_{450} of greater than or equal to 0.200 indicated a positive result. On a dual wavelength spectrophotometer, an $OD_{450/620}$ of less

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than 0.160 indicated a negative result and an OD_{450/620} of greater than or equal to 0.160 indicated a positive result.

A positive test result indicated the specimen contained elevated levels of lactoferrin when compared with a reference value established for healthy control subjects. A negative test result indicated the specimen did not contain elevated levels of lactoferrin relative to samples from healthy control subjects.

g. Results

One hundred forty-nine subjects having IBD were tested according to the above procedure. Seventy-seven of the subjects, or 51.7%, were male and seventy-two of them, or 48.3%, were female. The tested male to female ratio closely approximates the 1:1 ratio observed in the general IBD patient population. Ages of the subjects ranged from 3 years to 78 years and thirty-two subjects, or 22%, were 16 years of age or younger. Seventy-seven subjects, or 51.7%, had CD and seventy-two of them, or 48.3% had UC.

Thirty-one subjects having IBS were tested. Six of the subjects, or 19.3%, were male and twenty-five of them, or 80.7%, were female. The tested male to female ratio closely approximates the 1:3 ratio observed in the general IBS population. Ages of the subjects ranged from 19 years to 78 years.

Fifty-six healthy subjects also were tested as controls. Twenty-eight of the subjects, or 50%, were male and twenty-eight of them, or 50%, were female. Ages of the subjects ranged from infants to 79 years. A summary of the tested subject population is illustrated in Table 9.

Table 9: Summary of Subject Population

Summary of Subject Population	
Total number of IBD patients	149
No. Males	77
No. Females	72
Total number of patients with CD	77
No. Males	43
No. Females	34
Total number of patients with UC	72
No. Males	34
No. Females	38
Total number of patients with irritable bowel syndrome	31
No. Males	6
No. Females	25
Total number of healthy persons	56
No. Males	28
No. Females	28

- 5 Fecal specimens were collected from each enrolled subject and stored at -70°C until tested. Sample consistencies ranged from liquid to solid, numbers for which are illustrated in Table 10 for each subject group. As can be seen, forty-five of the IBD specimens were liquid specimens, sixty-two were semi-solid specimens, and forty-two were solid specimens. One of the IBS
- 10 specimens was a liquid specimen, thirteen were semi-solid specimens, and seventeen were solid specimens. All of the specimens from healthy control subjects were solid.

Table 10: Summary of Specimen Consistencies for Each Subject Group

Total number of IBD patients (CD and UC)	149
Total number of liquid specimens	45
Total number of semi-solid specimens	62
Total number of solid specimens	42
Total number of patients with IBS	31
Total number of liquid specimens	1
Total number of semi-solid specimens	13
Total number of solid specimens	17
Total number of healthy persons	56
Total number of liquid specimens	0
Total number of semi-solid specimens	0
Total number of solid specimens	56

5 The level of fecal lactoferrin in each specimen was determined using the qualitative lactoferrin ELISA as previously described. A specimen dilution of 1:400 was used. Results were reported as positive if an optical density of greater than or equal to 0.200 was observed. Conversely, results were reported as negative if an optical density of less than 0.200 was observed.

10 Of the IBD subject group, ninety-two subjects had active disease and fifty-seven had inactive disease. Of the active group, a total of eighty subjects, or 87.0%, tested positive in the assay. Of the inactive group, a total of thirty-two subjects, or 56.1%, tested positive. Of the forty-one subjects having active UC, a total of thirty-six subjects, or 87.8% tested positive in the assay. Of the fifty-one subjects having active CD, forty-four, or 86.3%, tested positive. All 15 thirty-one patients having active IBS and all fifty-six healthy control subjects tested negative in the assay. A summary of assay test results is illustrated in Table 11 and various individual comparisons are illustrated in Tables 12, 13 and 14, as more fully described below.

Table 11: Summary of ELISA test Results for CD, UC, Active IBS, and Healthy Control Subjects

Total IBD	149	75.2% (112)	24.8% (37)
Active	92	87.0% (80)	13.0% (12)
Inactive	57	56.1% (32)	43.0% (25)
Total CD	77	77.9% (60)	22.1% (17)
Active	56	86.3% (44)	13.7% (7)
Inactive	26	61.5% (16)	38.5% (10)
Total UC	72	72.2% (52)	27.7% (20)
Active	41	87.8% (36)	12.2% (5)
Inactive	31	51.6% (16)	48.4% (15)
Total Active IBS	31	0	100.0% (31)
Total Healthy Persons	56	0	100.0% (56)

- 5 When distinguishing samples from active IBD subjects from subject samples having IBS or from healthy control samples, the ELISA exhibited a sensitivity of 87% and specificity of 100%. Sensitivity was calculated by dividing the number of persons having IBD and testing positive in the ELISA by the number of subjects having IBD. Specificity was calculated by
- 10 dividing the number of subjects having IBD and testing positive in the ELISA by the number of subjects testing positive in the ELISA. The predictive positive and negative values were 100% and 87.9%, respectively, and the correlation was 93.3%. These results are summarized in Table 12.

Table 12: Statistical Evaluation using the ELISA to Distinguish Active IBD from IBS/Healthy Control Subjects

ELISA positive	80	0
ELISA negative	12	87

Sensitivity	87.0%
Specificity	100%
Predictive Positive Value	100%
Predictive Negative Value	87.9%
Correlation	93.3%

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When distinguishing samples from active UC subjects from subject samples having IBS or from healthy control subjects, the ELISA exhibited a sensitivity of 87.8% and a specificity of 100%. The predictive positive and negative values were 100% and 94.6%, respectively, and the correlation was 96.1%. These results are summarized in Table 13.

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Table 13: Statistical Evaluation using the ELISA to Distinguish Active UC from IBS/Healthy Control Subjects

ELISA positive	36	0
ELISA negative	5	87

Sensitivity	87.8%
Specificity	100%
Predictive Positive Value	100%
Predictive Negative Value	94.6%
Correlation	96.1%

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When distinguishing subject samples having active CD from subject samples having IBS or from healthy control samples, the ELISA exhibited a sensitivity of 86.3% and a specificity of 100%. The predictive

positive and negative values were 100% and 92.6%, respectively, and the correlation was 94.9%. These results are summarized in Table 14.

Table 14: Statistical Evaluation using the ELISA to Distinguish Active CD from IBS/Healthy Control Subjects

ELISA positive	44	0
ELISA negative	7	87

5

Sensitivity	86.3%
Specificity	100%
Predictive Positive Value	100%
Predictive Negative Value	92.6%
Correlation	94.9%

h. Reproducibility and Precision

The inter-assay variation was determined by analyzing eight lactoferrin-negative and eight lactoferrin-positive fecal specimens over a three day period. The average % Coefficient of Variation (CV) was 23.5% for the positive specimens and 7.4% for the negative specimens. The intra-assay variation was determined by analyzing twelve fecal specimens using six replicates in one lot of kits. The intra-assay analysis ranged in %CV from 2.7 to 24.0 with an average of 8.7%.

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Example 2 Lactoferrin Quantitative Assay

In the quantitative assay of the present invention, fecal specimens preferably are serially diluted ten-fold and added to microtiter wells containing immobilized polyclonal antibodies against human lactoferrin. If endogenous lactoferrin is present, it will bind to the antibodies during an incubation at approximately 37°C. Following the incubation, conjugate comprised of polyclonal antibodies coupled to horseradish peroxidase enzyme is added and allowed to bind to captured lactoferrin. Unbound conjugate is then washed from the well and a component substrate (e.g., tetra-methyl-benzidine and hydrogen

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peroxide) is added for color development. Following the substrate incubation, 0.6N sulfuric acid is added to quench the reaction and the absorbance or optical density (OD) is obtained spectrophotometrically at 450 nm on a single wavelength device. Fecal lactoferrin concentrations are determined by
5 comparison to a standard curve generated using purified human lactoferrin.

a. Preparation of Standard Curve

A 1 mg/mL stock solution of purified human lactoferrin, manufactured by Sigma Immunochemicals of St. Louis, Missouri, was prepared using 10 mg of lactoferrin dissolved in 10 mL of sterile phosphate buffered saline
10 (PBS) at pH 7.4. Serial two-fold dilutions of lactoferrin were made using the range of approximately 6 to 100 ng/mL in Diluent. For the analysis, 0.1 mL of each standard was assayed in duplicate. Optical densities (OD₄₅₀) were determined and plotted versus lactoferrin concentration to generate standard curves. The linear portion of the curve was determined by linear regression
15 analysis using the Log-Log method (Microsoft EXCEL, Microsoft R Office). The lowest dilution of specimen that gave an OD₄₅₀ within the linear portion of the curve was used to determine the lactoferrin concentration. The final concentration was obtained by multiplying the concentration by the dilution factor.

20 b. Quantitative Test Procedure

In order to assess the ability of the quantitative ELISA to measure the level of fecal lactoferrin, two fecal specimens collected six weeks apart from six female and five male adults were diluted and then spiked with lactoferrin to a concentration of 25 ng/mL. The estimated lactoferrin that was determined
25 represents the level of lactoferrin determined from a standard curve generated with the quantitative ELISA. The % Variation represents the difference between the actual amount used to spike the sample and the estimated amount. Under these conditions, the variations ranged from 1.0% to 85.8% for females and 8.8% to 47.0% for males. Results showed a higher percent variation in female adults
30 as compared to male adults. The stool samples that showed a higher variation had higher levels of lactoferrin prior to spiking. The results are illustrated in Tables 15 and 16 below.

Table 15. Stool samples of female adult subjects spiked to a final concentration of 25 ng/mL

Sample ID	Actual Concentration (ng/mL)	Estimated Concentration (ng/mL)	% Variation
1	25	15.4	38.4
2	25	22.9	8.5
3	25	21.8	12.7
4	25	28.4	13.5
5	25	16.2	35.3
6	25	15.8	37.0
7	25	35.5	41.8
8	25	46.5	85.8
9	25	27.7	10.8
10	25	32.3	29.1
11	25	26.1	4.3
12	25	25.3	1.0

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Table 16. Stool samples of male adult subjects spiked to a final concentration of 25 ng/mL

Sample ID	Actual Concentration (ng/mL)	Estimated Concentration (ng/mL)	% Variation
1	25	21.9	12.4
2	25	21.2	15.0
3	25	20.9	16.3
4	25	21.4	14.4
5	25	20.8	16.8
6	25	22.8	8.8
7	25	28.9	15.5
8	25	29.4	17.4
9	25	36.7	47.0
10	25	19.5	21.9

A second method for spiking was using the same two stool specimens collected six weeks apart from six female and five male adults were diluted and spiked with lactoferrin to a concentration of 4 $\mu\text{g/mL}$. The estimated lactoferrin represents the level of lactoferrin determined from a standard curve generated by the quantitative ELISA. The % Variation represents the difference between the actual amount used to spike the sample and the estimated value.

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Under these conditions, the variation ranged from 11.3% to 84.9% for females and from 5.0% to 39.2% for males. Results were similar to those obtained with specimens spiked with 25 ng/mL lactoferrin as described above, showing a higher percent variation in female adults compared to male adults. The results are illustrated in Tables 17 and 18 below.

Table 17. Stool samples of female adult subjects spiked to a final concentration of 4 µg/mL

1	4	4.5	11.3
2	4	4.6	15.3
3	4	5.3	33.4
4	4	4.9	21.4
5	4	3.5	11.5
6	4	3.4	14.7
7	4	5.3	32.7
8	4	6.7	67.6
9	4	5.5	38.6
10	4	5.8	44.9
11	4	5.8	43.9
12	4	7.4	84.9

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Table 18. Stool samples of male adult subjects spiked to a final concentration of 4 µg/mL

1	4	4.7	17.5
2	4	4.6	14.4
3	4	4.2	5.0
4	4	5.6	39.2
5	4	4.2	5.9
6	4	4.7	18.5
7	4	4.7	16.5
8	4	5.5	37.9
9	4	5.3	33.6
10	4	4.3	6.6

Monitoring Using the Quantitative ELISA

The quantitative ELISA of the present invention was used to follow the lactoferrin levels of single patient suffering from ulcerative colitis during a flare of active disease through remission. The patient showed extremely high levels of lactoferrin (e.g., 9749.37 µg/mL feces) during the peak of the active disease, the levels dropping rapidly (e.g., to 7.42 µg/mL feces) following anti-inflammatory drug therapy. Levels elevated dramatically again during a relapse and leveled at slightly above those of healthy control persons (e.g., 11.06 µg/mL feces) during periods of remission. Thus, lactoferrin levels determined according to the quantitative ELISA of the present invention accurately depicted disease activity in response to medical treatment.

Example 3

ASCA Assay

In this example, a fecal sample was obtained and serially diluted 20 fold. 100 µl of the diluted sample was added to a test well of a microassay plate coated with extract of *Saccharomyces cerevisiae*. The sample then was incubated at 37°C to allow antibodies to *Saccharomyces cerevisiae* to bind to the extract of *Saccharomyces cerevisiae*. Following incubation, anti-human Ig polyclonal antibodies coupled to horseradish peroxidase enzyme (conjugate) were added to the test well and allowed to bind to captured ASCA. Unbound conjugate then was washed from the well and one component substrate (tetramethyl-benzidine and hydrogen peroxide) was added for color development. Following the substrate incubation, 0.1M sulfuric acid was added to quench the reaction and the optical density (OD) was obtained spectrophotometrically at 450 nm using a single wavelength spectrophotometer.

The method described above was used in a clinical study to test a total of 86 IBD patients (55.8% males and 44.2% females). The approximate 1 to 1 ratio of males to females was similar to the ratio observed in IBD patient populations. The IBS patient group ranged in age from 19 to 78 years and was 9% male and 91% female. This ratio of males to females (1:10) reflects the increased incidence for IBS in females as seen in patient populations. The healthy control (HC) patient group ranged in age from 20 to 79 years old and was

33.3% male and 66.6% female. A summary of the patient population in the clinical study is shown in Table 19.

TABLE 19 Summary of patient population.

Summary of Clinical Histories (N=120)	Total Subjects
Total number of IBD patients	86
No. Males	48
No. Females	38
Total number of patients with Crohn's Disease	49
No. Males	26
No. Females	23
Total number of patients with ulcerative colitis	37
No. Males	22
No. Females	15
Total number of patients with irritable bowel syndrome	22
No. Males	2
No. Females	20
Total number of healthy controls	12
No. Males	4
No. Females	8

5

In the clinical study, there were 37 ulcerative colitis patients, 49 Crohn's disease patients, 22 irritable bowel patients, and 12 healthy controls. Fecal samples were collected from each enrolled subject and stored at -70°C until tested. The optical densities for each sample were determined using the method described above. Results were reported as positive for fecal ASCA if an optical density of greater than or equal to 0.200 was observed. Results were reported as negative for fecal ASCA if an optical density of less than or equal to 0.199 was observed. Other clinical data, such as stool consistency, was also determined. Table 20, below, contains the clinical data and test results for healthy patients that participated in this clinical study. Table 21, below, contains the clinical data and test results for patients with ulcerative colitis patients that participated in this clinical study. Table 22, below, contains the clinical data and test results for patients with Crohn's disease that participated in this study. Table 23, below, contains the clinical data and test results for patients with irritable bowel syndrome that participated in this study.

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TABLE 20 Clinical data and test results for healthy controls

Donor ID	Sex	Age Range	Previous of chronic GI illness	Stool Consistency	Optical Density	Fecal ASCA
HC1	F	40 - 49	NO	Solid	0.098	NEGATIVE
HC2	F	40 - 49	NO	Solid	0.089	NEGATIVE
HC3	M	70 - 79	NO	Solid	0.095	NEGATIVE
HC4	F	60 - 69	NO	Solid	0.085	NEGATIVE
HC5	M	70 - 79	NO	Solid	0.083	NEGATIVE
HC6	F	70 - 79	NO	Solid	0.076	NEGATIVE
HC7	F	50 - 59	NO	Solid	0.124	NEGATIVE
HC8	F	40 - 49	NO	Solid	0.095	NEGATIVE
HC9	F	50 - 49	NO	Solid	0.111	NEGATIVE
HC10	F	40 - 49	NO	Solid	0.111	NEGATIVE
HC11	M	50 - 60	NO	Solid	0.070	NEGATIVE
HC12	M	50 - 60	NO	Solid	0.054	NEGATIVE

5 TABLE 21 Clinical data and test results for ulcerative colitis patients

Patient ID	Sex	Age	Disease	Stool Consistency	Disease Activity	Optical Density	Fecal ASCA
UC1	F	46	UC	Liquid	ACTIVE	0.184	NEGATIVE
UC2	M	39	UC	Liquid	ACTIVE	0.378	POSITIVE
UC3	F	30	UC	Semi-Solid	ACTIVE	0.193	NEGATIVE
UC4	F	31	UC	Semi-Solid	INACTIVE	0.319	POSITIVE
UC5	F	30	UC	Semi-Solid	ACTIVE	0.114	NEGATIVE
UC6	M	61	UC	Semi-Solid	INACTIVE	0.115	NEGATIVE
UC7	F	68	UC	Liquid	INACTIVE	0.091	NEGATIVE
UC8	F	45	UC	Liquid	ACTIVE	0.356	POSITIVE
UC9	F	21	UC	Semi-Solid	ACTIVE	0.082	NEGATIVE
UC10	F	27	UC	Liquid	ACTIVE	0.161	NEGATIVE
UC11	F	24	UC	Solid	INACTIVE	0.104	NEGATIVE
UC12	F	74	UC	Semi-Solid	INACTIVE	0.091	NEGATIVE
UC13	M	69	UC	Semi-Solid	ACTIVE	0.070	NEGATIVE
UC14	M	19	UC	Solid	INACTIVE	0.088	NEGATIVE
UC15	M	62	UC	Solid	INACTIVE	0.054	NEGATIVE
UC16	F	70	UC	Solid	INACTIVE	0.056	NEGATIVE
UC17	M	23	UC	Liquid	ACTIVE	0.573	POSITIVE
UC18	F	52	UC	Solid	ACTIVE	0.073	NEGATIVE
UC19	M	60	UC	Solid	INACTIVE	0.062	NEGATIVE

UC20	F	52	UC	Liquid	ACTIVE	0.089	NEGATIVE
UC21	M	31	UC	Solid	INACTIVE	0.064	NEGATIVE
UC22	M	44	UC	Semi-Solid	INACTIVE	0.143	NEGATIVE
UC23	F	30	UC	Liquid	ACTIVE	0.110	NEGATIVE
UC24	M	48	UC	Semi-Solid	INACTIVE	0.096	NEGATIVE
UC25	F	37	UC	Liquid	ACTIVE	0.282	POSITIVE
UC26	F	32	UC	Solid	ACTIVE	0.107	NEGATIVE
UC27	F	46	UC	Liquid	ACTIVE	0.199	NEGATIVE
UC28	M	49	UC	Semi-Solid	INACTIVE	0.161	NEGATIVE
UC29	F	42	UC	Solid	INACTIVE	0.080	NEGATIVE
UC30	F	41	UC	Semi-Solid	INACTIVE	0.087	NEGATIVE
UC31	F	43	UC	Solid	INACTIVE	0.070	NEGATIVE
UC32	M	30	UC	Solid	ACTIVE	0.103	NEGATIVE
UC33	F	43	UC	Solid	INACTIVE	0.092	NEGATIVE
UC34	F	33	UC	Semi-Solid	INACTIVE	0.075	NEGATIVE
UC35	M	58	UC	Semi-Solid	ACTIVE	0.121	NEGATIVE
UC36	F	32	UC	Semi-Solid	ACTIVE	0.083	NEGATIVE

TABLE 22 Clinical Data and test results for Crohn's disease patients.

Patient ID	Sex	Age	Disease	Stool Consistency	Disease Activity	Optical Density	FECAL ASCA
CD1	M	26	CD	Liquid	INACTIVE	1.900	POSITIVE
CD2	M	60	CD	Liquid	ACTIVE	2.849	POSITIVE
CD3	F	66	CD	Liquid	ACTIVE	0.282	POSITIVE
CD4	F	74	CD	Semi-Solid	INACTIVE	0.091	NEGATIVE
CD5	F	25	CD	Solid	INACTIVE	0.162	NEGATIVE
CD6	F	66	CD	Semi-Solid	INACTIVE	1.240	POSITIVE
CD7	M	39	CD	No Data	ACTIVE	1.150	POSITIVE
CD8	F	46	CD	Liquid	ACTIVE	0.160	NEGATIVE
CD9	F	46	CD	Semi-Solid	INACTIVE	0.074	NEGATIVE
CD10	F	56	CD	Solid	ACTIVE	0.406	POSITIVE
CD11	M	56	CD	Solid	ACTIVE	0.168	NEGATIVE
CD12	F	56	CD	Liquid	ACTIVE	0.732	POSITIVE
CD13	M	21	CD	Solid	ACTIVE	1.369	POSITIVE
CD14	M	52	CD	Semi-Solid	INACTIVE	0.136	NEGATIVE
CD15	M	63	CD	Solid	INACTIVE	0.134	NEGATIVE
CD16	M	34	CD	Solid	ACTIVE	0.076	NEGATIVE
CD17	F	45	CD	Semi-Solid	ACTIVE	0.160	NEGATIVE
CD18	M	67	CD	Semi-Solid	INACTIVE	0.059	NEGATIVE
CD19	F	46	CD	No Data	ACTIVE	0.839	POSITIVE

CD20	M	66	CD	Semi-Solid	INACTIVE	0.084	NEGATIVE
CD21	M	63	CD	Liquid	ACTIVE	0.780	POSITIVE
CD21	M	51	CD	Semi-Solid	ACTIVE	3.000	POSITIVE
CD22	M	34	CD	Semi-Solid	ACTIVE	1.447	POSITIVE
CD23	M	21	CD	Solid	ACTIVE	2.757	POSITIVE
CD24	F	78	CD	Semi-Solid	INACTIVE	0.092	NEGATIVE
CD25	F	27	CD	Semi-Solid	ACTIVE	0.979	POSITIVE
CD26	M	40	CD	Liquid	ACTIVE	0.373	POSITIVE
CD27	M	51	CD	Liquid	ACTIVE	0.978	POSITIVE
CD28	M	42	CD	Liquid	ACTIVE	0.089	NEGATIVE
CD29	F	31	CD	Solid	INACTIVE	0.075	NEGATIVE
CD30	F	59	CD	Solid	ACTIVE	0.088	NEGATIVE
CD31	M	35	CD	Semi-Solid	ACTIVE	1.487	POSITIVE
CD32	M	37	CD	Semi-Solid	INACTIVE	1.257	POSITIVE
CD33	F	77	CD	Solid	INACTIVE	0.093	NEGATIVE
CD34	F	40	CD	No Data	ACTIVE	1.762	POSITIVE
CD35	F	38	CD	Liquid	ACTIVE	0.098	NEGATIVE
CD36	M	51	CD	Liquid	ACTIVE	2.326	POSITIVE
CD37	M	38	CD	Semi-Solid	ACTIVE	0.091	NEGATIVE
CD38	M	37	CD	Liquid	ACTIVE	0.372	POSITIVE
CD39	M	59	CD	Semi-Solid	ACTIVE	0.224	POSITIVE
CD40	F	41	CD	Solid	ACTIVE	0.503	POSITIVE
CD41	M	41	CD	Solid	ACTIVE	0.117	NEGATIVE
CD42	M	48	CD	Liquid	ACTIVE	0.115	NEGATIVE
CD43	F	40	CD	Solid	INACTIVE	0.638	POSITIVE
CD44	F	72	CD	Solid	ACTIVE	0.087	NEGATIVE
CD45	F	32	CD	Liquid	INACTIVE	0.911	POSITIVE
CD46	F	24	CD	Liquid	ACTIVE	0.341	POSITIVE
CD47	M	23	CD	Solid	INACTIVE	0.088	NEGATIVE
CD48	F	34	CD	Liquid	ACTIVE	0.599	POSITIVE

TABLE 23 Clinical data and test results for irritable bowel syndrome patients

Patient ID	Sex	Age	Disease	Stool consistency	Disease Activity	Optical Density	Fecal ASCA
IBS1	F	56	IBS	Semi-Solid	ACTIVE	0.132	NEGATIVE
IBS2	F	48	IBS	Solid	ACTIVE	0.103	NEGATIVE
IBS3	F	30	IBS	Solid	ACTIVE	0.073	NEGATIVE
IBS4	F	31	IBS	Solid	ACTIVE	0.074	NEGATIVE
IBS5	F	72	IBS	Semi-Solid	ACTIVE	0.079	NEGATIVE

IBS6	F	47	IBS	Solid	ACTIVE	0.088	NEGATIVE
IBS7	F	19	IBS	Semi-Solid	ACTIVE	0.105	NEGATIVE
IBS8	F	58	IBS	Semi-Solid	ACTIVE	0.107	NEGATIVE
IBS9	F	40	IBS	Solid	ACTIVE	0.065	NEGATIVE
IBS10	F	33	IBS	Semi-Solid	ACTIVE	0.065	NEGATIVE
IBS11	F	78	IBS	Solid	ACTIVE	0.071	NEGATIVE
IBS12	F	74	IBS	Semi-Solid	ACTIVE	0.063	NEGATIVE
IBS13	F	50	IBS	Semi-Solid	ACTIVE	0.052	NEGATIVE
IBS14	F	39	IBS	Solid	ACTIVE	0.079	NEGATIVE
IBS15	F	54	IBS	Solid	ACTIVE	0.080	NEGATIVE
IBS16	M	49	IBS	Semi-Solid	ACTIVE	0.238	POSITIVE
IBS17	M	53	IBS	Solid	ACTIVE	0.123	NEGATIVE
IBS18	F	34	IBS	Solid	ACTIVE	0.091	NEGATIVE
IBS19	F	43	IBS	Solid	ACTIVE	0.075	NEGATIVE
IBS20	F	35	IBS	Solid	ACTIVE	0.075	NEGATIVE
IBS21	F	51	IBS	Semi-Solid	ACTIVE	0.081	NEGATIVE
IBS22	F	40	IBS	Solid	ACTIVE	0.083	NEGATIVE

There were a total of 49 patients with Crohn's disease and 37 with ulcerative colitis. In the Crohn's disease group, a total of 55.1% patients were positive for fecal ASCA. In the ulcerative colitis group, 13.5% were positive. Of the 22 IBS patients, a single patient (4.6%) was positive for fecal ASCA. All 12 healthy controls were negative. A summary of positive results for fecal ASCA is shown in Table 24.

TABLE 24 Summary of positive results for Crohn's disease, ulcerative colitis, active IBS, and healthy controls

Total Assessments N = 120	Total	Fecal ASCA Positive	Fecal ASCA Negative
Total IBD (Crohn's disease and ulcerative colitis)	86	37.2% (32)	62.8% (54)
Total Crohn's Disease	49	55.1% (27)	44.9% (22)
Total Ulcerative Colitis	37	13.5% (5)	86.5% (32)
Total Active IBS	22	4.6% (1)	96.4% (21)
Total Healthy Controls	12	0	100.0% (12)

5

When distinguishing Crohn's disease from ulcerative colitis, fecal ASCA exhibited a sensitivity of 55.1% and specificity of 86.5%. The predictive positive and negative values were 84.4% and 59.3%, respectively, and the correlation was 68.6% as shown in Table 25.

10

TABLE 25 Statistical evaluation using the presence of fecal ASCA to distinguish Crohn's disease from ulcerative colitis

N=86	Crohn's disease	Ulcerative colitis
Fecal ASCA positive	27	5
Fecal ASCA negative	22	32

15

Sensitivity	55.1%
Specificity	86.5%
Predictive Positive Value	84.4%
Predictive Negative Value	59.3%
Correlation	68.6%

When distinguishing Crohn's disease from ulcerative colitis, irritable bowel syndrome and healthy controls, fecal ASCA exhibited a sensitivity of 55% and a specificity of 91.6%. The predictive positive and negative values were 82% and 75%, respectively, and the correlation was 77% as shown below in Table 26.

TABLE 26 Statistical evaluation using fecal ASCA to distinguish Crohn's disease from ulcerative colitis, irritable bowel syndrome/healthy controls

N=120	Crohn's disease	UC/IBS/Healthy Controls
Fecal ASCA positive	27	6
Fecal ASCA negative	22	65

10

Sensitivity	55.1%
Specificity	91.6%
Predictive Positive Value	81.8%
Predictive Negative Value	74.7%
Correlation	76.7%

The mean optical densities for each group were obtained and differences were tested for statistical significance using a two-tailed t-test giving a p-value result. Of the 33 patients that tested positive for fecal ASCA, there were 27 CD, 5 UC, and 1 IBS. Sensitivity, specificity and overall correlation were 55.1%, 91.5% and 76.7%, respectively. ASCA-positive CD showed a higher mean \pm SD A450 of 1.183 \pm 0.794 as compared to 0.382 \pm 0.113 for UC and the single A450 of 0.0091 \pm 0.0038 for IBS. There was a significant difference between CD and all other subject groups. A summary of the statistical analysis is listed in Table 27.

20

TABLE 27 Summary of the Mean and P values of Optical Densities for Fecal ASCA

Test Group	Mean Optical Density	Standard Deviation	Optical Density Range	P Value
CD	1.183	0.794	0.341-3.000	CD vs UC,IBS,HC P < 0.005
UC	0.382	0.113	0.382-0.113	CD vs UC P < 0.05
IBS	0.091	0.038	0.052-0.238	CD vs IBS P < 0.005
HC	0.091	0.019	0.054-0.124	CD vs HC P < 0.005

5 The sensitivity of the fecal ASCA test also was determined using serial two fold dilutions of highly purified ASCA antibodies. For the analysis, standard curves were generated using the kit diluent. The test was consistently positive at a concentration of 0.62 µg/mL as determined by a cutoff absorbency value of ≥ 0.200 . Individual results are shown below in Table 28. The standard

10 curves are shown in FIG. 1.

TABLE 28 Standard curves generated using purified ASCA antibodies

Purified ASCA Antibodies (µg/mL)	Test 1	Test 2	Mean	Std Dev
5.00	1.702	1.856	1.779	0.108
2.50	1.117	1.099	1.108	0.012
1.25	0.634	0.624	0.629	0.007
0.62	0.303	0.329	0.316	0.018
0.31	0.191	0.164	0.177	0.019
0.16	0.115	0.113	0.114	0.001
0.08	0.090	0.077	0.083	0.009
0.04	0.063	0.065	0.064	0.001

15 Tests also were conducted to determine what type of immunoglobulins (antibodies) were present in a fecal sample and in serum. The immunoglobulin typing was done for human IgA, human IgA_{sec}, human IgD, human IgM, and human IgG. The immunoglobulin typing was done on a fecal

sample from 6 Crohn's disease patients and 2 ulcerative colitis and on a serum control sample using pre-absorbed Ig-type specific conjugates. The serum control sample was obtained from a patient with a confirmed allergy to *Saccharomyces cerevisiae*.

- 5 Of the Crohn's disease patients, 5 patients exhibited a response to IgA and IgA_{sec}, 4 patients exhibited a response to IgM and a single patient exhibited a response to IgG. Of the 2 ulcerative colitis patients, a single patient reacted with the Ig conjugate. The serum control only exhibited a response to individual immunoglobulins IgM and IgG. A response to IgA and IgA_{sec} occurred the fecal samples but not in the control serum sample. A summary of results is shown in Table 29.

TABLE 29 A Summary of Immunoglobulin Typing of ASCA in a Human Fecal sample and a Serum Control

Patient Number	Disease	IgA Conjugate	IgA _{sec} Conjugate	IgD Conjugate	IgM Conjugate	IgG Conjugate	Ig Conjugate
1	Crohn's Disease	+	+	-	+	+	+
2	Crohn's Disease	+	+	-	+	-	+
3	Crohn's Disease	-	-	-	-	-	-
4	Crohn's Disease	+	+	NO DATA	+	-	+
5	Crohn's Disease	+	+	NO DATA	-	-	+
6	Crohn's Disease	+	+	NO DATA	+	-	+
7	UC	-	-	-	-	-	-
8	UC	-	-	-	-	-	+
Serum Control	Yeast Allergy	-	-	-	+	+	+

Example 4

ANCA ASSAY

The ANCA specific immunoassay was used to differentiate ulcerative colitis and other gastrointestinal illnesses such as Crohn's disease and irritable bowel syndrome by measuring the level of total fecal ANCA. A qualitative immunoassay such as an enzyme-linked immunoassay that utilizes both monoclonal and polyclonal antibodies to endogenous human ANCA indicated the absence or presence of ulcerative colitis. In the example qualitative assay, the fecal specimen was diluted 10 fold and added to a well containing immobilized neutrophil antigens. If ANCA was present, it was bound to the antigens during the incubation at 37°C. Following the incubation, anti-human Ig polyclonal antibodies coupled to horseradish peroxidase enzyme (conjugate) were added and allowed to bind to captured ANCA. Unbound conjugate was then washed from the well and one component substrate (tetramethybenzidine and hydrogen peroxide) was added for color development. Following the substrate incubation, 0.1M sulfuric acid was added to quench the reaction and the optical density (OD) was obtained spectrophotometrically at 450 nm.

Using the procedure described above, a total of 98 IBD patients were enrolled and comprised 51% males and 49% females with an age range of 0 to 69 years. The approximate 1 to 1 ratio is similar to the ratio observed in IBD patient populations. The IBS patient group had an age range of 5 to 39 years with 57% males and 43% females. The healthy controls were 55% male and 45% female and comprised the age range of 20 to 79 years. Individual numbers for each age group are shown in Table 30.

TABLE 30. Summary of patient population.

Summary of Clinical Histories (N=116)	Total Subjects
Total number of IBD patients	98
No. Males	50
No. Females	48
Total number of patients with Crohn's Disease	47
No. Males	26
No. Females	21
Total number of patients with ulcerative colitis	51
No. Males	24
No. Females	27
Total number of patients with irritable bowel syndrome	7
No. Males	4
No. Females	3
Total number of healthy persons	11
No. Males	6
No. Females	5

There were 51 ulcerative colitis patients, 47 Crohn's disease patients, 7 irritable bowel patients, and 11 healthy adults recruited for the study. Fecal specimens were collected from each enrolled patient and stored at -70°C until tested. Specimen consistency ranged from solid to liquid. The level of fecal ANCA was determined using the qualitative ANCA ELISA as previously described. Disease activity was defined using elevated fecal lactoferrin as an indicator of intestinal inflammation. A dilution of 1:10 was used in the ANCA-CHEK (qualitative ELISA) and results were reported as positive (absorbance values ≥ 0.140) or negative (absorbance values < 0.140). The mean optical densities, standard deviation and P values (two-tailed student T-test with unequal variance) were determined for the ANCA positive ulcerative colitis patients. Of the 26 patients that tested positive for fecal ANCA, there were 4 CD, 21 UC, and 1 healthy person. ANCA-positive UC showed a mean \pm SD OD₄₅₀ of 0.311 ± 0.166 . The mean OD for the UC patients was significantly different from IBS and healthy persons (p value < 0.0005). A summary of the statistical analysis is listed in Table 31.

TABLE 31. Summary of the mean, standard deviation and P values for ANCA-CHEK Optical densities

Group ID	Number	Mean Optical Density	Standard Deviation	Optical Density Range	P values
ANCA + UC	21	0.311	0.166	0.141-0.804	UC vs CD p<0.5
ANCA + CD	4	0.209	0.115	0.141-0.381	UC vs CD, IBS, H p<0.0005
IBS	7	0.078	0.027	0.047-0.121	UC vs CD, IBS p<0.005
Healthy	11	0.071	0.041	0.039-0.104	UC vs IBS, H p<0.0005

In the IBD group, there were 47 with Crohn's disease and 51 with ulcerative colitis. In the ulcerative colitis group, 41% were positive. In the Crohn's disease group, a total of 9% patients were positive by the ANCA-CHEK. Of the 11 healthy persons, 1 was positive and all 7 IBS patients were negative by the ANCA-CHEK test. A summary of positive results for the ANCA-CHEK is shown in Table 32 and individual results are listed in Tables 33 through 34.

10. TABLE 32. Summary of positive results for Crohn's disease, ulcerative colitis, and IBS

Total Assessments N = 116	Total	Fecal ANCA Positive	Fecal ANCA Negative
Total IBD (Crohn's disease and ulcerative colitis)	98	26% (25)	75% (73)
Total Crohn's Disease	47	9% (4)	91% (43)
Total Ulcerative Colitis	51	41% (21)	59% (30)
Total IBS	7	0	7
Total Healthy Persons	11	9%(1)	91%(10)

When distinguishing ulcerative colitis from Crohn's disease, the *ANCA-CHEK* exhibited a sensitivity of 41% and specificity of 92%. The predictive positive and negative values were 84% and 59%, respectively, and the correlation was 65% (Table 33).

5

TABLE 33. Statistical evaluation using the *ANCA-CHEK* to distinguish Crohn's disease from ulcerative colitis

N=98	Ulcerative colitis	Crohn's disease
<i>ANCA-CHEK</i> positive	21	4
<i>ANCA-CHEK</i> negative	30	43

Sensitivity	41%
Specificity	92%
Predictive Positive Value	84%
Predictive Negative Value	59%
Correlation	65%

10

When distinguishing ulcerative colitis from irritable bowel syndrome and healthy persons, the *ANCA-CHEK* exhibited a sensitivity of 41% and a specificity of 92%. The predictive positive and negative values were 81% and 67%, respectively, and the correlation was 70% (Table 34).

15

TABLE 34. Statistical evaluation using the *ANCA-CHEK* to distinguish ulcerative colitis from Crohn's disease, irritable bowel syndrome and healthy persons

N=116	Ulcerative colitis	Crohn's disease IBS/Healthy
<i>ANCA-CHEK</i> positive	21	5
<i>ANCA-CHEK</i> negative	30	60

20

Sensitivity	41%
Specificity	92%
Predictive Positive Value	81%
Predictive Negative Value	67%
Correlation	70%

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The sensitivity of the *ANCA-CHEK* was determined using serial two fold dilutions of human ANCA positive serum. For the analysis, standard curves were generated using the sample diluent. The test was consistently positive to a titer of 0.063 as determined by a cutoff absorbance value of ≥ 0.200 .

5 Individual results are shown below in Table 35 and standard curves are shown in FIG. 2.

TABLE 35. Standard curves generated using *ANCA-CHEK* (cut-offs are bolded)

Human ANCA Serum	Test 1	Test 2	Test 3	Mean	Std Dev
1.000 (Neat)	1.441	1.469	1.525	1.478	0.043
0.500	1.098	0.941	1.014	1.018	0.079
0.250	0.717	0.595	0.666	0.659	0.061
0.125	0.492	0.428	0.444	0.455	0.033
0.063	0.327	0.303	0.320	0.317	0.012
0.032	0.196	0.295	0.221	0.237	0.051
0.016	0.132	0.184	0.179	0.165	0.029
Diluent	0.067	0.093	0.109	0.090	0.021

10

Table 36, below contains the clinical data and test results for patients with ulcerative colitis that participated in the study. Table 37, below, contains the clinical data and test results for patients with Crohn's disease that participated in the study. Table 38, below, contains the clinical data and test results for patients with irritable bowel syndrome that participated in the study. Table 39, below, contains the clinical data and test results for healthy patients that participated in the study.

15

TABLE 36. Clinical and ELISA results for ulcerative colitis patients.

Patient ID	Sex	Age Range	Disease	Disease Activity	ANCA-CHEK OD ₄₅₀	ANCA-CHEK Result
UC1	F	10-19	UC	INACTIVE	0.053	NEGATIVE
UC2	F	5-9	UC	INACTIVE	0.107	NEGATIVE
UC3	F	5-9	UC	ACTIVE	0.058	NEGATIVE
UC4	M	10-19	UC	INACTIVE	0.048	NEGATIVE
UC5	M	10-19	UC	ACTIVE	0.512	POSITIVE
UC6	F	10-19	UC	ACTIVE	0.061	NEGATIVE
UC7	M	5-9	UC	ACTIVE	0.211	POSITIVE
UC8	M	10-19	UC	ACTIVE	0.106	NEGATIVE
UC9	M	10-19	UC	INACTIVE	0.804	POSITIVE
UC10	M	10-19	UC	ACTIVE	0.091	NEGATIVE
UC11	F	10-19	UC	ACTIVE	0.169	POSITIVE
UC12	F	10-19	UC	ACTIVE	0.209	POSITIVE
UC13	F	10-19	UC	ACTIVE	0.351	POSITIVE
UC14	F	10-19	UC	ACTIVE	0.198	POSITIVE
UC15	F	5-9	UC	ACTIVE	0.098	NEGATIVE
UC16	F	5-9	UC	ACTIVE	0.050	NEGATIVE
UC17	F	10-19	UC	ACTIVE	0.091	NEGATIVE
UC18	M	10-19	UC	ACTIVE	0.603	POSITIVE
UC19	M	10-19	UC	ACTIVE	0.091	NEGATIVE
UC20	F	10-19	UC	ACTIVE	0.142	POSITIVE
UC21	M	10-19	UC	ACTIVE	0.074	NEGATIVE
UC22	F	10-19	UC	ACTIVE	0.105	NEGATIVE
UC23	M	10-19	UC	INACTIVE	0.256	POSITIVE
UC24	F	0-4	UC	ACTIVE	0.308	POSITIVE
UC25	F	5-9	UC	ACTIVE	0.072	NEGATIVE
UC26	M	10-19	UC	INACTIVE	0.237	POSITIVE
UC27	M	10-19	UC	ACTIVE	0.048	NEGATIVE
UC28	M	10-19	UC	ACTIVE	0.049	NEGATIVE
UC29	M	10-19	UC	ACTIVE	0.059	NEGATIVE
UC30	F	10-19	UC	INACTIVE	0.047	NEGATIVE
UC31	M	10-19	UC	ACTIVE	0.055	NEGATIVE
UC32	M	10-19	UC	INACTIVE	0.044	NEGATIVE
UC33	F	10-19	UC	ACTIVE	0.043	NEGATIVE
UC34	M	5-9	UC	ACTIVE	0.046	NEGATIVE
UC35	M	10-18	UC	INACTIVE	0.043	NEGATIVE
UC36	M	10-17	UC	INACTIVE	0.040	NEGATIVE

UC37	F	10-19	UC	ACTIVE	0.047	NEGATIVE
UC38	F	0-4	UC	ACTIVE	0.049	NEGATIVE
UC39	F	5-9	UC	INACTIVE	0.363	POSITIVE
UC40	F	10-19	UC	INACTIVE	0.046	NEGATIVE
UC41	M	10-19	UC	ACTIVE	0.118	NEGATIVE
UC42	F	50-59	UC	ACTIVE	0.230	POSITIVE
UC43	M	10-19	UC	ACTIVE	0.051	NEGATIVE
UC44	F	30-39	UC	ACTIVE	0.060	NEGATIVE
UC45	F	50-59	UC	ACTIVE	0.465	POSITIVE
UC46	M	50-59	UC	ACTIVE	0.274	POSITIVE
UC47	F	30-39	UC	ACTIVE	0.141	POSITIVE
UC48	M	60-69	UC	ACTIVE	0.184	POSITIVE
UC49	F	40-49	UC	ACTIVE	0.397	POSITIVE
UC50	F	40-49	UC	ACTIVE	0.337	POSITIVE
UC51	M	30-39	UC	ACTIVE	0.143	POSITIVE

TABLE 37. Clinical and ELISA results for Crohn's disease patients.

Patient ID	Sex	Age Range	Disease	Disease Activity	ANCA-CHEK OD ₄₅₀	ANCA-CHEK Result
CD1	M	10-19	CD	ACTIVE	0.050	NEGATIVE
CD2	M	10-19	CD	ACTIVE	0.113	NEGATIVE
CD3	M	10-19	CD	ACTIVE	0.050	NEGATIVE
CD4	F	10-19	CD	ACTIVE	0.381	POSITIVE
CD5	F	10-19	CD	ACTIVE	0.058	NEGATIVE
CD6	M	10-19	CD	INACTIVE	0.068	NEGATIVE
CD7	M	10-19	CD	ACTIVE	0.066	NEGATIVE
CD8	M	5-9	CD	ACTIVE	0.059	NEGATIVE
CD9	F	10-19	CD	ACTIVE	0.059	NEGATIVE
CD10	F	10-19	CD	ACTIVE	0.065	NEGATIVE
CD11	F	10-19	CD	INACTIVE	0.055	NEGATIVE
CD12	M	10-19	CD	INACTIVE	0.071	NEGATIVE
CD13	F	10-19	CD	ACTIVE	0.065	NEGATIVE
CD14	M	10-19	CD	ACTIVE	0.098	NEGATIVE
CD15	F	10-19	CD	ACTIVE	0.099	NEGATIVE
CD16	M	10-19	CD	ACTIVE	0.166	POSITIVE
CD17	F	10-19	CD	ACTIVE	0.147	POSITIVE
CD18	M	10-19	CD	ACTIVE	0.057	NEGATIVE
CD19	F	10-19	CD	ACTIVE	0.084	NEGATIVE
CD20	M	10-19	CD	ACTIVE	0.053	NEGATIVE

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CD21	F	10-19	CD	ACTIVE	0.074	NEGATIVE
CD22	M	10-19	CD	ACTIVE	0.054	NEGATIVE
CD23	M	0-5	CD	ACTIVE	0.055	NEGATIVE
CD24	M	10-19	CD	ACTIVE	0.067	NEGATIVE
CD25	M	10-19	CD	ACTIVE	0.099	NEGATIVE
CD26	M	5-9	CD	ACTIVE	0.086	NEGATIVE
CD27	F	10-19	CD	ACTIVE	0.043	NEGATIVE
CD28	F	10-19	CD	ACTIVE	0.064	NEGATIVE
CD29	M	5-9	CD	INACTIVE	0.039	NEGATIVE
CD30	M	10-19	CD	ACTIVE	0.071	NEGATIVE
CD31	F	10-15	CD	ACTIVE	0.109	NEGATIVE
CD32	M	10-19	CD	INACTIVE	0.057	NEGATIVE
CD33	M	10-19	CD	ACTIVE	0.141	POSITIVE
CD34	M	10-19	CD	INACTIVE	0.045	NEGATIVE
CD35	F	10-19	CD	ACTIVE	0.051	NEGATIVE
CD36	F	10-19	CD	ACTIVE	0.132	NEGATIVE
CD37	F	10-19	CD	INACTIVE	0.046	NEGATIVE
CD38	M	10-19	CD	ACTIVE	0.057	NEGATIVE
CD39	F	20-29	CD	INACTIVE	0.051	NEGATIVE
CD40	F	20-29	CD	ACTIVE	0.053	NEGATIVE
CD41	M	50-59	CD	ACTIVE	0.060	NEGATIVE
CD42	F	50-59	CD	ACTIVE	0.062	NEGATIVE
CD43	M	20-29	CD	ACTIVE	0.056	NEGATIVE
CD44	F	60-69	CD	ACTIVE	0.130	NEGATIVE
CD45	M	60-69	CD	ACTIVE	0.078	NEGATIVE
CD46	F	40-49	CD	ACTIVE	0.116	NEGATIVE
CD47	M	60-69	CD	ACTIVE	0.057	NEGATIVE

TABLE 38. Clinical and ELISA results for Irritable bowel syndrome patients.

Patient ID	Sex	Age Range	Disease	ANCA-CHEK OD ₄₅₀	ANCA-CHEK Results
IBS1	F	10-19	IBS	0.056	NEGATIVE
IBS2	M	10-19	IBS	0.047	NEGATIVE
IBS3	M	5-9	IBS	0.099	NEGATIVE
IBS4	M	10-19	IBS	0.068	NEGATIVE
IBS5	M	10-19	IBS	0.092	NEGATIVE
IBS6	F	20-29	IBS	0.121	NEGATIVE
IBS7	F	30-39	IBS	0.064	NEGATIVE

TABLE 39. Clinical and ELISA results for healthy persons.

Subject ID	Sex	Age Range	ANCA-CHEK OD ₄₅₀	ANCA-CHEK Results
D1	F	40-49	0.087	NEGATIVE
D2	M	20-29	0.078	NEGATIVE
D5	M	20-29	0.178	POSITIVE
D15	M	50-59	0.041	NEGATIVE
D17	M	50-59	0.039	NEGATIVE
D18	F	40-49	0.069	NEGATIVE
D19	F	60-69	0.050	NEGATIVE
D20	M	70-79	0.039	NEGATIVE
D21	F	70-79	0.104	NEGATIVE
D22	M	60-69	0.045	NEGATIVE
D24	F	50-59	0.054	NEGATIVE

5 In summary, the present invention is directed to a method for the differentiation of inflammatory bowel disease (IBD) from irritable bowel disease (IBS) followed by distinguishing ulcerative colitis and Crohn's disease from other gastrointestinal illnesses. This highly differential method first uses the presence of elevated fecal lactoferrin as a marker of intestinal inflammation to

10 differentiate IBD from IBS. Patients suspected of IBD are then analyzed for fecal anti-*Saccharomyces cerevisiae* antibodies (ASCA) as an indicator of Crohn's disease and fecal anti-neutrophil cytoplasmic antibodies (ANCA) as an indicator of ulcerative colitis. IBD patients are further monitored for intestinal inflammation using fecal lactoferrin to evaluate the effectiveness of medical

15 therapy and predict relapse. The apparatus consists of either a qualitative enzyme-linked immunoassay or other immunoassay that utilizes antibodies specific to total endogenous lactoferrin, ASCA and ANCA in human feces.

The method and apparatus may be used by healthcare providers to identify IBD and distinguish ulcerative colitis and Crohn's disease from other

20 gastrointestinal illnesses. The present invention has been described in relation to particular embodiments, which are intended in all respects to be illustrative rather than restrictive. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its scope.

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From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects hereinabove set forth together with other advantages which are obvious and which are inherent to the method.

It will be understood that certain features and subcombinations are
5 of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims.

CLAIMS

What the invention claimed is:

1. A method for testing a sample from a person, the method comprising: obtaining a fecal sample from a person; determining whether
5 lactoferrin is present in the sample; if so, determining whether anti-*Saccharomyces cerevisiae* antibodies (ASCA) and anti-neutrophil cytoplasmic antibodies (ANCA) are present in the sample.
2. The method of claim 1, wherein the presence of elevated lactoferrin is used to aid in the diagnosis of inflammatory bowel disease.
- 10 3. The method of claim 2, wherein the absence of elevated lactoferrin is used to aid in the diagnosis of irritable bowel syndrome.
4. The method of claim 3, wherein if the sample contains anti-neutrophil cytoplasmic antibodies, a diagnosis of ulcerative colitis may be substantially concluded.
- 15 5. The method of claim 4, wherein if the sample contains anti-*Saccharomyces cerevisiae* antibodies a diagnosis of Crohn's disease may be substantially concluded.
6. The method of claim 3, wherein the presence of anti-neutrophil cytoplasmic antibodies is used to aid in the differentiation of ulcerative
20 colitis from Crohn's disease.
7. The method of claim 4, wherein the presence of anti-*Saccharomyces cerevisiae* antibodies is used to aid in the differentiation of Crohn's disease from ulcerative colitis.
8. The method of claim 1, wherein the lactoferrin, anti-
25 *Saccharomyces cerevisiae* antibodies and anti-neutrophil cytoplasmic antibodies are measured using one of enzyme-linked immunoassays, lateral flow membrane tests and immunoassays utilizing antibodies or capturing fragments.

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9. The method of claim 1, wherein the presence of lactoferrin is measured determined by a qualitative ELISA.
10. The method of claim 1, wherein the presence of lactoferrin is measured quantitatively.
- 5 11. The method of claim 1, further comprising: diluting the sample.
12. The method of claim 11, further comprising: contacting the sample with immobilized polyclonal antibodies to endogenous lactoferrin to create a treated sample.
- 10 13. The method of claim 12, further comprising: contacting said treated sample with enzyme-linked polyclonal antibodies to create a readable sample.
14. The method of claim 13, further comprising: determining the optical density of said readable sample at 450 nm.
- 15 15. The method of claim 14, further comprising: generating a purified lactoferrin standard curve.
16. The method of claim 15, further comprising: comparing said optical density of said readable sample to said standard curve to determine the concentration of endogenous lactoferrin in said the sample.
- 20 17. The method of claim 11, further comprising: contacting the sample with antigens of *Saccharomyces cerevisiae* to create a treated sample.
18. The method of claim 17, further comprising: contacting the treated sample with polyvalent antibodies to human immunoglobulin conjugated to an enzyme to create a readable sample.
- 25 19. The method of claim 18, further comprising: determining the optical density of the readable sample.

- 45 -

20. The method of claim 11, further comprising: contacting the sample with neutrophil cytoplasmic antigens to create a treated sample.

21. The method of claim 20, further comprising: contacting the treated sample with polyvalent antibodies to human immunoglobulin to create
5 a readable sample.

22. The method of claim 21, further comprising: determining an optical density of the readable sample at 450 nm.

23. The method of claim 22, wherein the presence of anti-neutrophil cytoplasmic antibodies is used to aid in the differentiation of ulcerative
10 colitis from Crohn's disease and other gastrointestinal illnesses.

24. A method for distinguishing inflammatory bowel disease from irritable bowel syndrome and for differentiating ulcerative colitis from Crohn's disease, the method comprising: obtaining a sample from a person; determining whether lactoferrin is present in the sample; if so, determining anti-
15 *Saccharomyces cerevisiae* antibodies (ASCA) and anti-neutrophil cytoplasmic antibodies (ANCA) are present in the sample, diagnosing the person with irritable bowel syndrome if elevated lactoferrin is not present in the sample; diagnosing the person with inflammatory bowel disease if lactoferrin is present in the sample; diagnosing the person with Crohn's disease if anti-*Saccharomyces*
20 *cerevisiae* antibodies are present in the sample; and diagnosing the person with ulcerative colitis if anti-neutrophil cytoplasmic antibodies are present in the sample.

25. The method of claim 24, wherein the sample is feces.

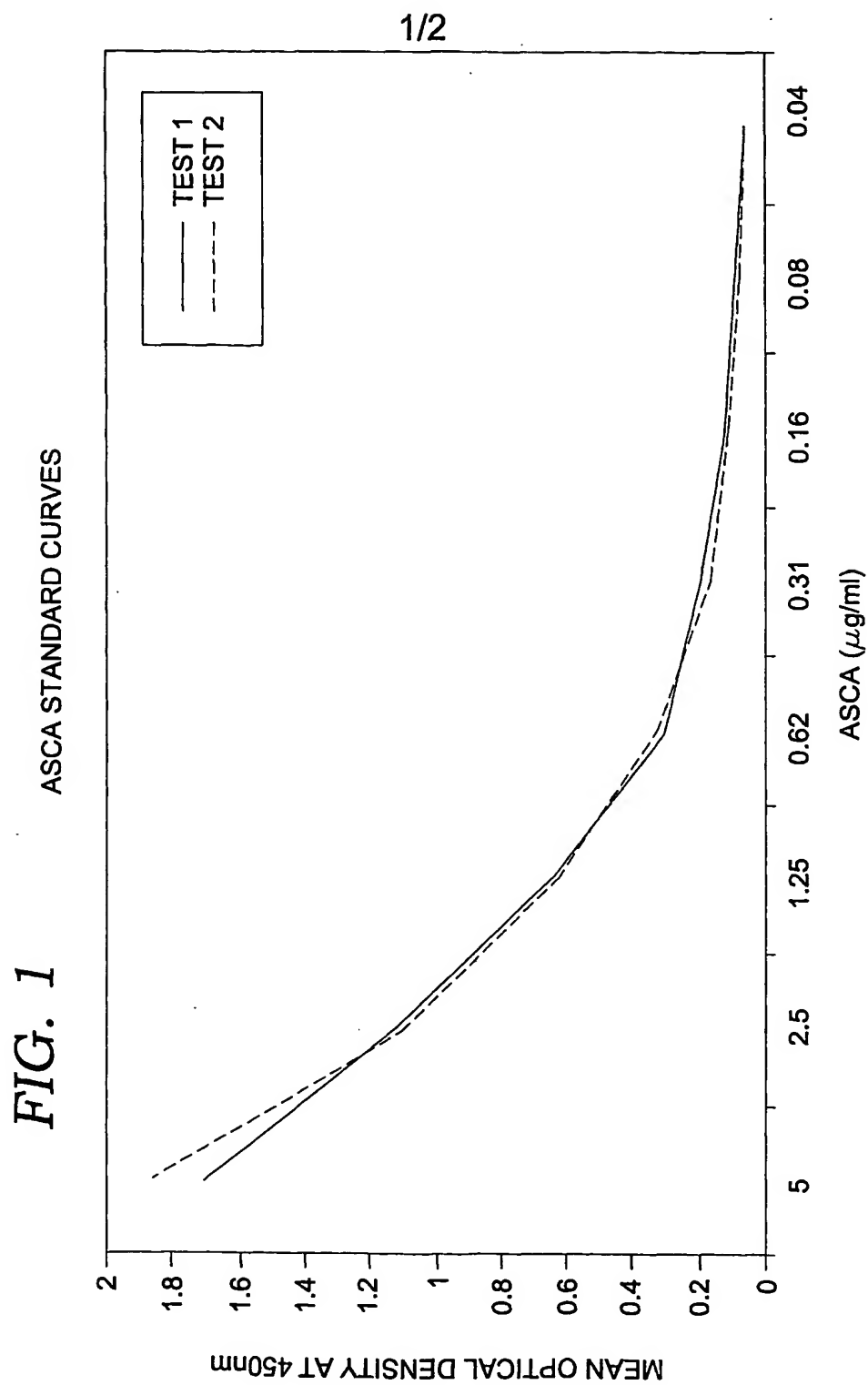
26. The method of claim 24, wherein the sample is one of
25 whole blood, serum, plasma, saliva, mucosal secretions, bodily fluid and bodily tissue.

27. The method of claim 24, wherein if lactoferrin is present in the sample, monitoring the person for changing levels of lactoferrin as an indicator for the effectiveness of medical therapy.

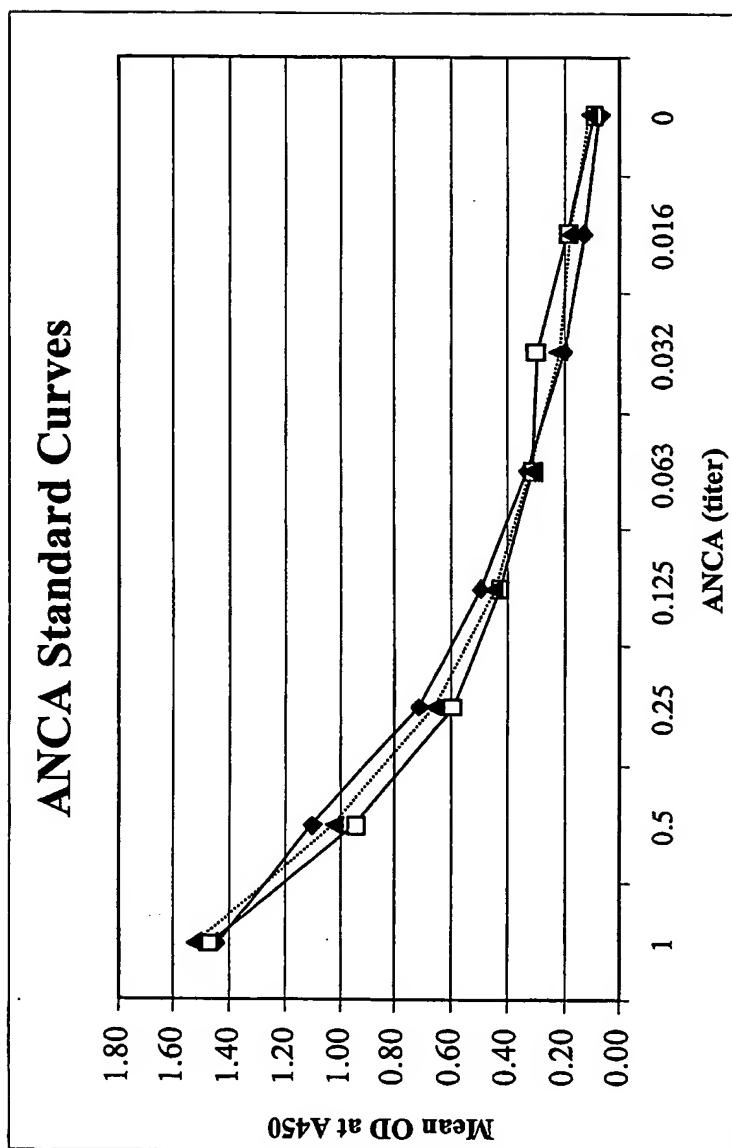
- 46 -

28. A kit for testing a fecal sample from a person to be diagnosed, the kit comprising: a plurality of microassay plates, wherein at least one plate contains immobilized polyclonal antibodies to human lactoferrin, at least one plate contains neutrophil cytoplasmic antigens and at least one plate
5 contains antigen of *Saccharomyces cerevisiae*; enzyme-linked polyclonal antibody to human lactoferrin; polyvalent antibodies to human immunoglobulin; enzyme substrate for color development.

29. The kit as recited in claim 28, further comprising: a stop solution for quenching the reaction.

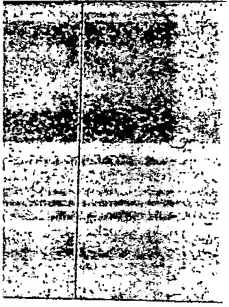
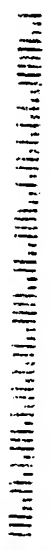


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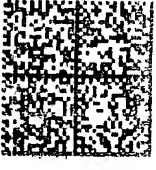
*FIG. 2*

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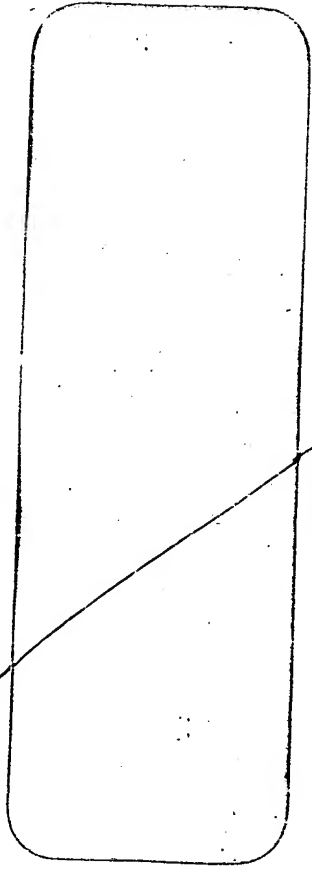
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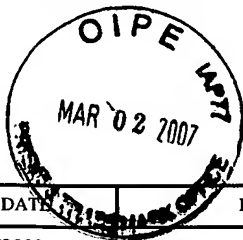
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/693,377	10/24/2003	James Hunter Boone	TLAB.100292	1630

7590 02/22/2007
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EXAMINER	
PORTNER, VIRGINIA ALLEN	
ART UNIT	PAPER NUMBER
1645	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	02/22/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

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Office Action Summary	Application No. 10/693,377	Applicant(s) BOONE ET AL.	
	Examiner Ginny Portner	Art Unit 1645	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 06 November 2006.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-27 is/are pending in the application.
- 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-27 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|--|---|
| 1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892) | 4) <input type="checkbox"/> Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____ |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) | 5) <input type="checkbox"/> Notice of Informal Patent Application |
| 3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date <u>2, 5/04; 7/06</u> . | 6) <input type="checkbox"/> Other: _____ |

DETAILED ACTION

Claims 1-27 are pending.

Election/Restrictions

1. Applicant's election without traverse of Group I, claims 1-27 in the reply filed on November 6, 2006 is acknowledged.

Claim Rejections - 35 USC § 112

2. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

3. Claims 1-27 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Claim 1-3 are directed to the measurement of fecal lactoferrin in any person, including breast fed infants. In light of evidence provided by US Pat. 6,727,073 which teaches that "fecal lactoferrin found in breast-fed infants is not an effective indicator of an inflammatory intestinal condition, as the presence of lactoferrin from breast milk will lead to false positives." The instantly claimed methods that diagnose inflammatory bowel disease and irritable bowel syndrome in any person, even breast fed infants are not enabled for the claimed invention as the method would result in false positive results.

The Wands factors to be considered:

.the quantity of experimentation necessary: undue due to false positives resulting from fecal lactoferrin originating from sources other than inflammatory neutrophils^{1,2} in breast fed infants and other types of lactoferrin associated diseases and disorders (see Levay et al, 1995, Table 7, page 262);

.the amount of direction or guidance presented: does not provide guidance to exclude

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false positive patients;

.the presence or absence of working examples: there are working examples, but determination of lactoferrin in breast fed infants is not described/claimed in such a way as to differentiate between various inflammatory bowel diseases;

.the nature of the invention: unpredictable in light of multiple sources of fecal lactoferrin can cause false positives in a patient;

.the state of the prior art: still developing with respect to which markers clearly determine/distinguish various forms of inflammatory bowel disease (see Bossuyt (2006, who shows variability in the presentation of markers in patients with inflammatory bowel disease, see entire article, where control patients present with autoantibodies to neutrophilic antigens (see Table 3, page 173; as well as ASCA healthy controls (see Table 4, page 175); Glaffer 1991 teaches IgA anti-ASCA antibodies are increased while IgG antibodies to ASCA are not; Oshitani et al (2001) further show only IgG4 is increased and not IgG1, G2 and G3 are not increased in inflammatory bowel disease.

.the relative skill of those in the art: high (immunoassay methods of determining an analyte);

.the predictability or unpredictability of the art: unpredictable in light of false positives due to breast fed infants would evidence elevated fecal lactoferrin levels and Bossuyt showing negative control patients to present with autoantibodies to neutrophil antigens and *Saccharomyces cerevisiae* (see page 173, Table 3 and page 175, Table 4);

.breadth of the claims: broad.

4. The following is a quotation of the second paragraph of 35 U.S.C. 112:

5. The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

6. Claims 1-27 provides for the use of lactoferrin (see claim 1 “is present”; claims 2-3, 6-7, 23 “is used”; claims 4-6 “may be”; 8-10: “are measured” , “is measured” etc.), but, since the claims do not set forth any active, positive steps delimiting how this use is actually practiced.

7. Claims 17-19 and 20-22 recite method steps of adding antigens to the sample of claims 11 and 1, but the methods of detecting anti-*Saccharomyces cerevisiae* antibodies and anti-neutrophil cytoplasmic antibodies are optionally set forth in claims 1 and 11 by the recitation “if so”. The methods of claims 17-19 and 20-22 are optional methods steps to only be carried out when the lactoferrin level in the patient sample of claim 1 is positive, the samples of claims 17-22 are not defined to be positive, and therefore set forth a combination of claim limitations that

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are only optionally carried out. The recitation of additional optional methods steps does not further limit the optional method of claims 1 and 11. The recitation of "further comprises" additional optional methods steps, does not set forth positively recited methods steps for the claimed methods.

8. Claims 13-14 rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claims 13-16 create a readable sample by contacting the treated sample with enzyme-linked polyclonal antibodies, but how the antibodies create the readable sample is not clearly nor distinctly claimed, in light of the critical and essential binding specificity of the polyclonal antibodies is not claimed. See In re Mayhew.

9. Claims 13-14, 17-18 and 21-23 are rejected under 35 U.S.C. 112, second paragraph, as being incomplete for omitting essential elements, such omission amounting to a gap between the elements. See MPEP § 2172.01. The omitted elements are:

10. the polyvalent antibodies to human immunoglobulin labeled with horseradish peroxidase added to a diluted fecal sample that is readable at 450 nm (claims 13-14 and 21-23).

11. a substrate must be added to the fecal samples in order for the enzyme in the enzyme-linked immunoglobulin/antibody complex to generate an emission spectra which is then read. No substrates for the recite enzymes of claims 13-14, 17-18 and 21-23) have been added to the samples.

12. The polyclonal antibodies of claims 21-23 do not comprise an enzyme and therefore what is readable in the treated sample is unclear and incomplete. The claims are incomplete by

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omitting an essential enzyme/substrate for producing a readable sample. Additionally, claim 22 determines an optical density at 450 nm, but no reagents or components in the fecal sample are positively recited as comprising an emission spectra of 450 nm in the diluted fecal sample.

What components in the fecal sample are readable at 450 nm? An essential element is missing from the claim that would be readable at 450 nm. See *In re Mayhew*.

13. Claims 13-14, 18-19 and 21-23 are rejected under 35 U.S.C. 112, second paragraph, as being incomplete for omitting essential steps, such omission amounting to a gap between the steps. See MPEP § 2172.01. The omitted steps are: forming antigen/antibody complexes specific to lactoferrin, *Saccharomyces cerevisiae* antigens and neutrophil cytoplasmic antigens and removing any non-specific human immunoglobulin not contained in the specific antigen/antibody complexes, adding anti-human immunoglobulin antibodies labeled with an enzyme; adding enzyme substrate to produce a readable sample. The methods as now claimed detect any human immunoglobulin that is readable and fecal samples are known to contain a plurality of immunoglobulins that are not directed to lactoferrin, *Saccharomyces cerevisiae* antigens and neutrophil cytoplasmic antigens. The methods as now claimed are not directed to specifically detected only those antibodies or human immunoglobulins that are antigen specific for the antigens recited in claim 1.

14. Claims 15-16 recite the limitation "purified lactoferrin" in dependence upon claims 1, 11-14. Claims 1, 11-14 do not recite any purification steps, and the only source of lactoferrin

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recited in the claims is the fecal sample of claim 1, which may or may not contain lactoferrin.

There is insufficient antecedent basis for this limitation in the claim

Claim Rejections - 35 USC § 102

15. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

16. Claims 1,8-12, 13, 15 and 24-25 are rejected under 35 U.S.C. 102(b) as being anticipated by Guerrant et al (US Pat. 5,124,252).

Instant claim 1 and 24: Guerrant et al disclose the instantly claimed invention directed to a method, the method comprising the steps of:

17. obtaining a fecal sample from a person (see abstract);

18. determining whether lactoferrin is present in the sample (three additional control specimens tested on 7 different occasions were all negative (see col. 3, lines 63-64).

Instant claim 8-10: wherein the presence of lactoferrin is measured by ELISA (see col. 4, lines 23-60, especially lines 30-31).

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Instant claim 11: further comprising diluting the sample (see col. 2, lines 38-42 “mixed with an equal amount of 0.1% Triton-X”).)

Instant claim 12: further comprising contacting the sample with immobilized polyclonal antibodies “Bacto-latex beads were coated with rabbit anti-human lactoferrin” and this latex bead suspension was added to “22 fecal specimens (see col. 4, lines 1-11), the endogenous lactoferrin being released by the leukocytes (see col. 4, lines 12-16 “children with diarrhea in the northeast of Brazil”).

Instant claim 13: further comprising contacting said treated sample with enzyme linked polyclonal antibodies to create a readable sample (see col. 4, lines 42-44 “peroxidase conjugation”, read both visually and spectrophotometrically (see col. 4, line 49 and claim 4).

While the reference is silent with respect to whether the rabbit antibodies are polyclonal or monoclonal antibodies, it is clear that the reference does not discuss nor describe the production of hybridoma and monoclonal production, therefore the antibodies are conventional rabbit sera that comprise polyclonal antibodies.

Instant claim 15: further comprising generating a purified lactoferrin standard curve (see col. 4, lines 29-32, varying concentrations of lactoferrin were coated in the wells). The sensitivity of the assay was 0.001 ug/ml or less lactoferrin (see claim 4). Guerrant et al (US Pat. 5,124,252) anticipates the instantly claimed invention that does not require the claimed method to measure anything more than endogenous lactoferrin when the sample when the lactoferrin determination is considered negative in light of all the claims reciting the phrase “if so”, which makes the following methods steps optional. Guerrant et al (US Pat. 5,124,252) anticipates the instantly claimed invention as now claimed.

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19. Claims 1-3, 11-12 and 24-26, 27 are rejected under 35 U.S.C. 102(b) as being anticipated by Fine et al (AJG, 1998).

Instant claim 1-3 and 24-27: Fine et al disclose the instantly claimed invention directed to a method, the method comprising the steps of:

20. obtaining a fecal sample from a person (see page 1301, col. 2, first two paragraphs);

21. determining whether lactoferrin is present in the sample (see page 1302, Table 1 “Diagnoses in 92 Patients with a negative fecal lactoferrin Test”, one patients test changed levels upon repeating the lactoferrin determination (see page 1302, Table 1, bottom of ledger narrative). The lactoferrin data was used to distinguish the patients that have inflammatory bowel disease or syndrome from those patients that have another bowel condition (see page 1302, Table 1).

Instant claim 11: further comprising diluting the sample (see page 1301, col. 2, paragraph 3).

Instant claim 12: further comprising contacting the sample with immobilized polyclonal antibodies latex beads were coated with rabbit anti-human lactoferrin, the endogenous lactoferrin is detected with the immobilized polyclonal antibodies. While the reference is silent with respect to whether the rabbit antibodies are polyclonal or monoclonal antibodies, it is clear that the reference does not discuss nor describe the production of hybridoma cell lines and monoclonal antibody production, therefore the antibodies are present in conventional rabbit sera that comprise polyclonal antibodies.

Fine et al anticipates the instantly claimed invention that does not require the claimed method to measure anything more than endogenous lactoferrin when the sample when the lactoferrin determination is considered negative in light of all the claims reciting the phrase “if

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so”, which makes the following methods steps optional. Fine et al anticipates the instantly claimed invention as now claimed.

22. Claims 24 and 26 are rejected under 35 U.S.C. 102(b) as being anticipated by Martins et al (1995). Martins et al disclose a method that comprises the step of :

Obtaining a whole blood, saliva, sputum (mucosal secretion sample) and gingival swabs (bodily fluid) from a patient (see abstract);

Determining whether lactoferrin is present in the sample (see abstract, negative for lactoferrin as an inflammatory marker in 7 individuals with healthy gums and teeth ; 4 edentulous patients were negative (see Figure 1 and 2, page 764). Martins et al anticipates the instantly claimed invention as now claimed.

Claim Rejections - 35 USC § 103

23. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

24. Claims 1-10, 24-26 are rejected under 35 U.S.C. 103(a) as being unpatentable over Nielsen et al (2000) in view of Targan et al (1995) and Fine (PG-Pub 2001/0036639A1, filing date March 2, 2001).

Nielsen et al describe biological activity markers of Inflammatory Bowel Disease (see title, page 359), wherein the markers include fecal lactoferrin (see page 360, col. 2, paragraph 1),

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and autoantibodies known as ANCA and ASCA (see page 361, col. 1-2). Nielson et al teach the methods step of :

Obtaining a fecal sample from a patient and determining the presence of fecal lactoferrin (see page 360, col. 2, paragraph 1) in order to provide for both sensitive and bowel-specific markers of disease and further determining the presence or absence of ASCA and ANCA in the patient (see page 361, col. 1-2).

Nielson et al teach the importance of assessing disease activity in inflammatory bowel disease (IBD), to include ulcerative colitis and Crohn's disease based upon clinical parameters and various biological disease markers (see page 359, col. 1, abstract, first sentence), but differs from the instantly claimed invention by failing to determine ANCA and ASCA in the fecal sample.

Targan et al (1995) teach ANCA antibodies are presenting mucosal lesions of the bowel (whole abstract; and page 3266, col. 2, paragraph 1) in ulcerative colitis patients (non-serum samples, see table II, page 3265; p3264, Figure 1, Table 1; diluted 1:2 (see page 3264, Results section, first paragraph) in an analogous art for the purpose of quantitatively (see Table 1, page 3264, col. 5) defining pANCA production is a consequence of a mucosal immune response associated with ulcerative colitis (full last sentence of abstract; Fig. 1, p 3264). Fine et al (20010036639) teach a method of measuring fecal antibodies directed to *Saccharomyces cerevisiae* (ASCA) (see claims 1, 19-21 and 43; [0054]) in an analogous art for the purpose of determining the presence of antibodies associated with diseases or disorders of the bowel, to include diagnosis of irritable bowel syndrome (see page 3, [0020] and [0015; 0018, entire paragraph, as well as second half of paragraph. "diarrhea"]).

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It would have been obvious to the person of ordinary skill in the art at the time the invention was made to measure fecal lactoferrin, ANCA and ASCA in a patient fecal sample because Nielson et al teach biological markers associated with inflammatory bowel disease, and teach fecal lactoferrin, as well as ANCA and ASCA to provide insight into disease activity associated with inflammatory bowel disease (see Nielsen et al, abstract, page 360, col. 2, p.1 and page 361, col. 1-2) and Targan et al and Fine et al teach the presence of ANCA and ASCA markers, respectively, are present in fecal/mucosal bowel samples and could be measured in the patient fecal sample along with the fecal lactoferrin determination. The person of ordinary skill in the art would have been motivated to determine fecal lactoferrin, along with fecal ANCA and ASCA markers because Nielsen et al teach that the lactoferrin is a measure of active bowel disease and measurement of ANCA and ASCA provide for differential diagnosis of the patient's type of inflammatory bowel disease (see Nielsen et al, page 361, col. 2, paragraph 3).

In the absence of a showing of unexpected results, the person of ordinary skill in the art would have been motivated by the reasonable expectation of success of determining the presence or absence of inflammatory bowel disease (see page 360, col. 1, p. 1) by determining the fecal lactoferrin test, a marker for active bowel inflammatory disease, as taught by Nielsen et al, and if positive, further determining the presence and amount of ANCA and ASCA antibodies in the fecal sample because Nielsen et al teach that the "combined measurement of pANCA and ASCA may be used advantageously in the sub-classification of IBD patients with indeterminate colitis. Both antibody specificities are measured by traditional quantitative solid phase immunosorbent assays, and they are highly specific (>90%) for both UC and CD with disease sensitivity around 50% in both cases (see Nielsen et al, page 361, col. 2, paragraph 3)."

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Nielsen et al in view of Targen et al and Fine et al obviate the instantly claimed invention as now claimed.

Conclusion

25. The prior art made of record and not relied upon is considered pertinent to applicant's disclosure. US005552292A is cited to show fecal lactoferrin as a marker for colorectal cancer (abstract).
26. Kayazawa et al (Feb. 2002) is cited to show measurement of lactoferrin in whole gut lavage fluid as a marker for disease activity in inflammatory bowel disease.
27. Peen et al (1993) is cited to show anti-lactoferrin antibodies and other types of ANCA in ulcerative colitis, PSC and Crohn's diseases.
28. Saitoh et al (page 3518) is cited to show fecal lactoferrin to be a useful marker for the presence of minimal intestinal inflammation in UC and CD, and elevated in almost all patients with active UC, indicating that bleeding and mucosal neutrophil infiltration are common features of all patients with UC.
29. Tribble et al (2001) is cited to show the measurement of stool lactoferrin as a marker for intestinal inflammation (see abstract and page 463, col. 2).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ginny Portner whose telephone number is (571) 272-0862. The examiner can normally be reached on flextime, but usually M-F, alternate Fridays off.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jeffrey Siew can be reached on (571) 272-0787. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Vgp
January 9, 2007



MARK NAVARRO
PRIMARY EXAMINER

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

7/06

Application Number	09981248 10/693,377 187
Filing Date	2001-10-16 2/c1
First Named Inventor	James Hunter Boone
Art Unit	1645
Examiner Name	
Attorney Docket Number	TLAB.100292

GP	1	KAZUO UCHIDA, et al., Immunochemical Detection of Human Lactoferrin in Feces as a New Marker for Inflammatory Gastrointestinal Disorders and Colon Cancer, Clinical Biochemistry, Vol. 27, No. 4, pp. 259-264, 1994, The Canadian Society of Clinical Chemists.	<input type="checkbox"/>
GP	2	OSAMU SAITOH, M.D., et al., Fecal Eosinophil Granule-Derived Proteins Reflect Disease Activity in Inflammatory Bowel Disease, The American Journal of Gastroenterology, Vol. 94, No. 12, 1999	<input type="checkbox"/>

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)

James Hunter Boone, et al.)

Serial No. 10/693,377)

Art Unit:)

Filed: October 24, 2003)

Examiner:)

Title: INFLAMMATORY BOWEL)
DISEASE AND IRRITABLE)
BOWEL SYNDROME IBD-)
FIRST CHEK DIAGNOSTIC)
PANEL)

Attorney Docket: TLAB.100292

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Dear Sir:

In compliance with Applicants duty of disclosure pursuant to 37 C.F.R. Section 1.56, it is requested that this Information Disclosure Statement be entered and that the references listed below and on the enclosed Form PTO-1449 be considered by the Examiner and made of record. Copies of the above-referenced documents are enclosed.

	U.S. PATENT NUMBER	ISSUE DATE	INVENTOR
GP	5,124,252	June 23, 1992	Guerrant, et al.
GP	5,359,038	October 25, 1994	Padron, Eloy
GP	5,455,160	October 3, 1995	Fagerhol, et al.

/Virginia Portner/

01/08/2007

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GP	5,552,292	September 3, 1996	Uchida, et al.
GP	5,932,429	August 3, 1999	Targan, et al.
GP	5,968,741	October 19, 1999	Plevy, et al.
GP	6,008,335	December 28, 1999	Rotter, et-al.
GP	6,218,129	April 17, 2001	Walsh, et al.

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EXAMINER INITIAL	PUBLICATION NUMBER	PUBLICATION DATE	COUNTRY OR PATENT OFFICE	CLASS	SUBCLASS
GP	WO 99/60403	25 November 1999	PCT	G01N 33/564, 33/569	
GP	WO 97/39356	23 October 1997	PCT	G01N 33/564	
GP	WO 98/46997	22 October 1998	PCT	G01N 33/53, C12Q 1/68	
GP	WO 01/11334 A2	15 February 2001	PCT	G01N	

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Respectfully submitted,

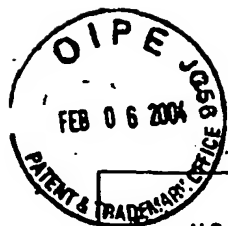


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Attorney Docket No. TLAB.100292



PATENT
Serial No. 10/693,377

PTO-1449 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	Attorney Docket No.: TLAB.100292	Serial Number: 10/693,377
Applicant: James Hunter Boone, et al.		
Title: INFLAMMATORY BOWEL DISEASE AND IRRITABLE BOWEL SYNDROME IBD- FIRST CHEK DIAGNOSTIC PANEL	Filing Date: October 24, 2003	Group:

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	PATENT NUMBER	ISSUE DATE	INVENTOR	CLASS	SUBCLASS	FILING DATE
GP	5,124,252	June 23, 1992	Guerrant, et al.			
GP	5,359,038	October 25, 1994	Padron, Eloy			
GP	5,455,160	October 3, 1995	Fagerhol, et al.			
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GP	5,968,741	October 19, 1999	Plevy, et al.			
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PATENT
Serial No. 10/693,377

FOREIGN PATENT OR PUBLISHED FOREIGN PATENT APPLICATION

EXAMINER INITIAL	DOCUMENT NUMBER	PUBLICATION DATE	COUNTRY OR PATENT OFFICE	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
GP	WO 99/60403	11/25/1999	PCT		G01N 33/564, 33/569		
GP	WO 97/39356	10/23/1997	PCT		G01N 33/564		
GP	WO 98/46997	10/22/1998	PCT		G01N 33/53, C12Q 1/68		
GP	WO 01/11334 A2	02/15/2001	PCT		G01N		
GP							

EXAMINER	/Virginia Portner/	DATE CONSIDERED	01/08/2007
EXAMINER: Initial citation if reference was considered. Draw line through citation if not in conformance to MPEP 609 and not considered. Include copy of this form with next communication to applicant.			

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EXAMINER INITIAL	DOCUMENT NUMBER	PUBLICATION DATE	COUNTRY OR PATENT OFFICE	CLASS	SUBCLASS
GP	WO 02/39883 A2 (TECHLAB, INC.)	23 May 2002	PCT	A61B	
GP	WO 01/36975 A1 (BINAX, INC.)	25 May 2001	PCT	G01N 33/553, 33/543	
GP	WO 92/16843 A1 (GUERRANT, R.L. et al.)	01 October 1992	PCT	G01N 33/545, 33/546	

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GP	DUBINSKY, M.C. et al. Clinical Utility of Serodiagnostic Testing in Suspected Pediatric Inflammatory Bowel Disease. American Journal of Gastroenterology (United States). March 2001, Vol. 96, No. 3, pages 758-765, especially abstract "the combination of these serodiagnostic test could maximize diagnostic accuracy and minimize invasive investigations," and entire document.
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/Virginia Portner/

01/08/2007

The Commissioner is hereby authorized to charge any additional fees that are required, or credit any overpayment, to Deposit Account No. 19-2112.

Respectfully submitted,

Jean M. Dickman

Jean M. Dickman
Reg. No. 48,538

PATENT
Serial No. 10/693,377

JMD:nlm

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816/474-6550

Attorney Docket No. TLAB.100292



PTO - 1449 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT BY APPLICANT	Attorney Docket No.: TLAB.100292	Serial Number: 10/693,377
	Applicant: James Hunter Boone; David Maxwell Lyerly; Tracy Dale Wilkins	
	Filing Date: October 24, 2003	Group:

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EXAMINER INITIAL	PATENT NUMBER	PUBLICATION NO	ISSUE DATE	INVENTOR	CLASS	SUB- CLASS	FILING DATE

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GP	BOONE, J. H. et al. Measurement of Anti-Saccharomyces cerevisiae antibodies in human feces as an indicator of Crohn's disease. American Journal of Gastroenterology. September 2002, Vol. 97, No. 9, page S253 (Abstract only).
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First Hit

L7: Entry 7 of 12

File: PGPB

Nov 14, 2002

DOCUMENT-IDENTIFIER: US 20020168698 A1

TITLE: Method for differentiating irritable bowel syndrome from inflammatory bowel disease (IBD) and for monitoring persons with IBD using total endogenous lactoferrin as a marker

CLAIMS:

1. A method for distinguishing irritable bowel syndrome from inflammatory bowel disease, the method comprising: obtaining a fecal sample from a person to be diagnosed; and determining whether said sample contains an elevated level of endogenous lactoferrin, wherein if said sample does contain an elevated level of endogenous lactoferrin, diagnoses of irritable bowel syndrome and other noninflammatory etiologies are substantially precluded.
2. The method as recited in claim 1, further comprising diluting said fecal sample.
3. The method as recited in claim 2, wherein said step of diluting said fecal sample comprises diluting said sample to a 1:400 dilution factor.
4. The method as recited in claim 1, wherein said endogenous lactoferrin comprises total lactoferrin from one or more of plasma, bile, leukocytes and mucosal secretions.
5. The method as recited in claim 1, wherein said endogenous lactoferrin is qualitatively determined.
6. The method as recited in claim 1, wherein said step of determining whether said sample contains an elevated level of endogenous lactoferrin includes contacting said sample with immobilized polyclonal antibodies to human lactoferrin to create a treated sample.
7. The method as recited in claim 6, wherein said step of determining whether said sample contains an elevated level of endogenous lactoferrin further includes contacting said treated sample with enzyme-linked polyclonal antibodies to create a readable sample.
8. The method as recited in claim 7, wherein said step of determining whether said sample contains an elevated level of endogenous lactoferrin further includes determining an optical density of said readable sample at 450 nm, wherein said optical density corresponds to a level of endogenous lactoferrin in the sample.
9. The method as recited in claim 8, wherein if said optical density of said readable sample is greater than 0.200, said fecal sample contains an elevated level of endogenous lactoferrin.
10. An assay for determining the concentration of endogenous lactoferrin, said assay comprising: obtaining a human fecal sample; diluting said fecal sample; contacting said sample with immobilized polyclonal antibodies to endogenous lactoferrin to create a treated sample; contacting said treated sample with enzyme-linked polyclonal antibodies to create a readable sample; determining the optical

density of said readable sample at 450 nm; generating a purified lactoferrin standard curve; and comparing said optical density of said readable sample to said standard curve to determine the concentration of endogenous lactoferrin in said fecal sample.

11. The assay as recited in claim 10, wherein said step of diluting said fecal sample comprises diluting said sample by serial ten-fold dilutions.

12. A diagnostic assay for differentiating irritable bowel syndrome from inflammatory bowel disease by determining the level of endogenous lactoferrin, said assay comprising: obtaining a human fecal sample; diluting said sample; contacting said sample with immobilized polyclonal antibodies to endogenous lactoferrin to create a treated sample; contacting said treated sample with enzyme-linked polyclonal antibodies to create a readable sample; and determining the optical density of said readable sample at 450 nm to determine whether said readable sample contains an elevated level of endogenous lactoferrin as compared to a reference value for healthy control subjects.

14. The diagnostic assay as recited in claim 13, wherein if said optical density of said readable sample is greater than or equal to 0.200, said fecal sample contains an elevated level of endogenous lactoferrin as compared to a reference value for healthy control subjects.

17. A kit for distinguishing irritable bowel syndrome from inflammatory bowel disease by testing a fecal sample from a person to be diagnosed, the kit comprising: one or more microassay plates, each said plate containing immobilized polyclonal antibodies to human lactoferrin; enzyme-linked polyclonal antibody to human lactoferrin; and enzyme substrate for color development.

20. A method for monitoring a patient having inflammatory bowel disease, the method comprising: obtaining a first fecal sample from the inflammatory bowel disease patient at a first time; determining the concentration of endogenous lactoferrin in said first fecal sample to obtain a first lactoferrin concentration; obtaining a second fecal sample from the inflammatory bowel disease patient at a second time later than said first time; determining the concentration of endogenous lactoferrin in said second sample to obtain a second lactoferrin concentration; and comparing said first lactoferrin concentration to said second lactoferrin concentration to evaluate any differences therebetween.

Attachment
H

IgG subclasses of anti *Saccharomyces cerevisiae* antibody in inflammatory bowel disease

N. Oshitani, F. Hato, Y. Jinno, Y. Sawa, S. Nakamura, T. Matsumoto, S. Seki, S. Kitagawa and T. Arakawa

Osaka City University Medical School, Osaka, Japan

Abstract

Background Elevation of serum anti *Saccharomyces cerevisiae* antibody (ASCA) has been reported in patients with Crohn's disease. We analysed the subclasses of Immunoglobulin (Ig) G reaction in ASCA in sera from patients with inflammatory bowel disease, healthy controls, and patients with intestinal Behçet's disease.

Materials and methods Serum samples were obtained from 29 patients with Crohn's disease, 30 patients with ulcerative colitis, 7 patients with intestinal Behçet's disease, and 12 healthy controls. Serum IgG subclasses IgG1, IgG2, IgG3, and IgG4 of ASCA were analysed using ELISA.

Results IgG4 ASCA was significantly increased in patients with inflammatory bowel disease. In patients with intestinal Behçet's disease, IgG1, IgG3, and IgG4 ASCA were increased.

Conclusions Differential responses, in terms of subclasses in ASCA, were found in patients with inflammatory bowel disease and patients with intestinal Behçet's disease, which may represent different pathophysiologies of these intestinal inflammatory diseases.

Keywords ASCA, Crohn's disease, immunoglobulin, *Saccharomyces cerevisiae*
Eur J Clin Invest 2001; 31(3): 221–225

Introduction

Immunological disorders are involved, concurrently influenced by genetic predisposition, in the pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD). However, the etiology of the disease is yet to be resolved. Abnormal immunological responses against diet antigens have been reported in patients with inflammatory bowel disease (IBD) [1–4], and food allergy may be contributed to the pathogenesis of IBD. Recently, increased serum titers of Immunoglobulin (Ig)G and IgA anti *Saccharomyces cerevisiae* antibody (ASCA) in patients with CD have been reported [5–8]. A combination of ASCA and perinuclear antineutrophil cytoplasmic antibody (p-ANCA) might be valuable in the differential diagnosis of patients with CD and UC [9]. As *S. cerevisiae* strains are widely used in baking and brewery, and elimination of yeast from diet may

have beneficial effects in the activity of CD [10], the pathogenetic role of ASCA in patients with CD is unknown.

Heterogeneous reactions of IgG subclasses have been reported that may depend on various conditions such as reaction to food antigen, to infectious agents, or autoimmune response (reviewed in [11]). Therefore, analysis of IgG subclass reactions may provide further information to the pathogenesis of ASCA generation in inflammatory diseases of the intestine.

Behçet's disease is a multi-system inflammatory disease, of unknown etiology, which is characterized by mucocutaneous, ocular, articular, vascular, gastrointestinal, and neurologic involvement. The prevalence of the Behçet's disease is higher in the Silk Road countries, and a high frequency of gastrointestinal involvement has been reported in Japanese patients [12]. Gastrointestinal lesions in both Crohn's disease and intestinal Behçet's disease often develop in the ileocecal region.

We studied the IgG subclasses of ASCA in patients with IBD, patients with intestinal Behçet's disease, and in healthy controls, to compare whether there is any different reactivity concerning IgG subclasses of ASCA among these subjects.

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Received 16 May 2000; accepted 5 November 2000

Patients and methods

Detection of serum ASCA by enzyme linked immunosorbent assay (ELISA)

Sera were obtained from 29 patients with UC (17 females, 12 males; mean age 35 years; range 20–55 years), 30 patients with CD (15 females, 15 males; mean age 32 years; range 18–67 years), 7 patients with Behçet's disease having intestinal involvement, either ileum or colon, or both (2 females, 5 males; mean age 36 years; range 16–53 years), and 12 healthy volunteers as controls (6 females, 6 males; mean age 32 years; range 25–51 years). Diagnosis of IBD was confirmed by clinical features and radiological, endoscopic, and histological features [13]. Diagnosis of Behçet's disease was performed according to the diagnostic criteria by the International Study Group for Behçet's Disease [14]. There were 9 total colitis and 20 left-sided colitis diagnoses in the 29 patients with UC, 10 were active and 19 were in remission. Nine patients with UC were taking steroids, and 23 were taking either sulphasalazine or 5-aminosalicylate derivatives. Of 30 patients with CD, 6 patients had small bowel involvement, 14 had small and large bowel involvement, and 10 had large bowel involvement only. Eight patients were taking steroids, and 16 patients were receiving dietary therapy at the study centre.

A 100 g sample of wet *S. cerevisiae* cells (bakers' yeast, Oriental Yeast, Tokyo, Japan) was washed and resuspended in sterile 0.15 M saline and heated at 100 °C for 1 h. It was then centrifuged at 2000 g for 10 min. The supernatant was removed and filtered (0.22 µm millipore). The extract was diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at a concentration of 10 µg mL⁻¹, determined by optical absorption measurement at 280 and 260 nm [15].

Each of microtiter plates (Multi Well Plate for ELISA, Sumitomo Bakelite Co. Ltd., Tokyo, Japan) were coated with 100 µL of *S. cerevisiae* extract at 37 °C for 1 h, then overnight at room temperature. The wells were washed three times with 0.01 M phosphate buffered saline (pH 7.3) with 0.01% Triton X-100 (PBS-T). Test sera were diluted 1 : 100 in 0.01 M PBS containing 0.1% bovine serum albumin, and assayed in duplicate. Diluted sera samples were applied 100 µL per well and incubated at 37 °C for 1 h. The wells were washed three times with 0.01 M PBS-T. A 100 µL sample per well of peroxidase conjugated mouse monoclonal antibodies to human IgG subclasses (IgG1, clone HP6070; IgG2, clone HP6014; IgG3, clone HP6047; IgG4, clone HP6023; Caltag Laboratories, Burlingame, CA, USA) were applied at a concentration of 1 : 1000 dilution in 0.01 M PBS containing 0.1% bovine serum albumin at 37 °C for 1 h. This was followed by three washes in 0.01 M PBS-T. The peroxidase substrate solution was freshly prepared; *o*-phenylenediamine (400 µg mL⁻¹) in 0.2 M phosphate-citrate buffer, pH 5.0, containing 0.02% H₂O₂. The colour reaction was terminated with 100 µL per well 4 M H₂SO₄, and the optical absorbance of each well was measured at 492 nm.

Antibody binding was expressed by binding index (BI) calculated as follows: (A_{492nm} (test serum) – A_{492nm} (background)) / (A_{492nm} (reference negative) – A_{492nm} (background)) (6).

Statistical analysis

Results were expressed as median (25th, 75th percentiles) and analysed by one-way analysis of variance with Bonferroni's correction. Differences were considered to be significant when the *P*-value was less than 0.05.

The study was approved by the Local Ethical Committee, Osaka City University Medical School, Osaka, Japan.

Results

BI of ASCA

IgG1 BI of ASCA (Fig. 1) was: 0.95 (0.66, 1.47) in healthy controls; 1.27 (0.71, 1.83) in patients with UC; 1.73 (0.85, 2.32) in patients with CD; and 3.00 (2.28, 8.88) in patients with intestinal Behçet's disease. IgG1 BI of ASCA was significantly increased in patients with intestinal Behçet's disease compared with healthy controls, patients with UC, and patients with CD. There were no statistical differences between IgG2 BI of ASCA between

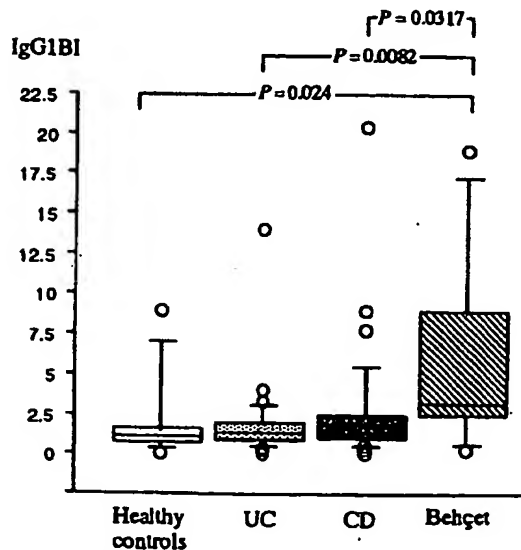


Figure 1 Binding index (BI) of IgG1 ASCA. IgG1 BI was significantly increased in patients with intestinal Behçet's disease compared with healthy controls, UC, and CD. UC, ulcerative colitis; CD, Crohn's disease; Behçet, intestinal Behçet's disease; BI, binding index. Bars show the 10th and 90th percentiles. Circle show data points below the 10th percentile and above the 90th percentile.

healthy controls, patients with UC, patients with CD, and patients with intestinal Behçet's disease (Table 1).

IgG3 BI of ASCA (Fig. 2) was: 0.67 (0.30, 1.13) in healthy controls; 1.20 (0.74, 1.77) in patients with UC; 0.90 (0.50, 1.25) in patients with CD; and 1.50 (1.50, 3.20) in patients with intestinal Behçet's disease. IgG3 BI of ASCA was significantly increased in patients with intestinal Behçet's disease compared with healthy controls, patients with UC, and patients with CD.

IgG4 BI of ASCA (Fig. 3) was: 1.14 (0.93, 1.19) in healthy controls; 2.83 (2.00, 3.51) in patients with UC; 2.00 (1.16, 2.88) in patients with CD; and 4.34 (1.00, 7.25) in patients with intestinal Behçet's disease. IgG4 BI of ASCA was significantly increased in patients with UC, patients with CD, and patients with intestinal Behçet's disease compared with healthy controls. It was significantly increased in patients with intestinal Behçet's disease compared with patients with UC, and patients with CD.

Patients taking sulphasalazine or 5-amino salicylate had significantly lower IgG3 BI but not in other subclasses; IgG3 BI was 2.00 (1.41, 2.31) in patients with UC not taking sulphasalazine or 5-aminosalicylate, and it was 1.01 (0.71, 1.30) in patients with UC taking the drug ($P = 0.0484$). IgG3 BI was 1.00 (0.73, 1.78) in patients with CD not taking sulphasalazine or 5-aminosalicylate, and it was 0.75 (0.16, 1.20) in patients with CD taking the drug ($P = 0.04$). CD patients with small bowel involvement (with or without large bowel involvement) had significantly higher IgG3 BI than patients only affected in the large bowel: IgG3 BI was 1.09 (0.71, 1.50) in patients with small bowel involvement, and 0.500 (0.0681) in patients with large bowel involvement ($P = 0.0135$). Age, gender, activity of the disease, extent of the disease in UC (left-sided or total colitis), and whether in receipt of dietary therapy or not in patients with CD did not affect any of the ASCA tested.

Discussion

Increased titers of serum IgG ASCA were found in patients with CD compared with patients with UC and

Table 1 Changes in IgG subclass in various conditions

	IgG1	IgG2	IgG3	IgG4
Th1 [25]		1	1	
Th2 [25]	1			
Chronic inflammation [16,20]				1
Dietary antibodies [26,27]				
UC associated p-ANCA [21,22]	1		1	
Vasculitis associated p-ANCA [22]				1
Anti-tropomyosin antibody [19]	1			
Control ASCA*	0.95	0.78	0.67	1.14
UC associated ASCA*	1.27	1.17	1.20	2.83
CD associated ASCA*	1.73	1.38	0.90	2.00
Intestinal Behçet associated ASCA*	3.00	2.00	1.50	4.34

*Median values of ASCA binding index.

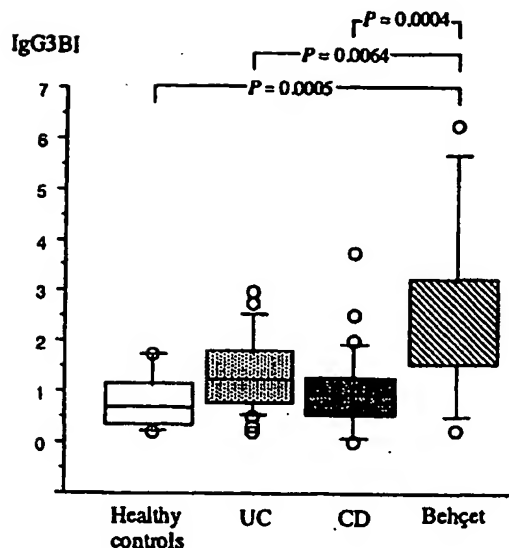


Figure 2 Binding index (BI) of IgG3 ASCA. IgG3 BI was significantly increased in patients with intestinal Behçet's disease compared with healthy controls, UC, and CD.

healthy controls [5–9]. However, antigenic heterogeneity in terms of ASCA reactivity has been reported [16]. Increased serum ASCA is characteristic, although not pathognomonic, in patients with CD, and conflicting results have been reported of IgG ASCA titers in patients

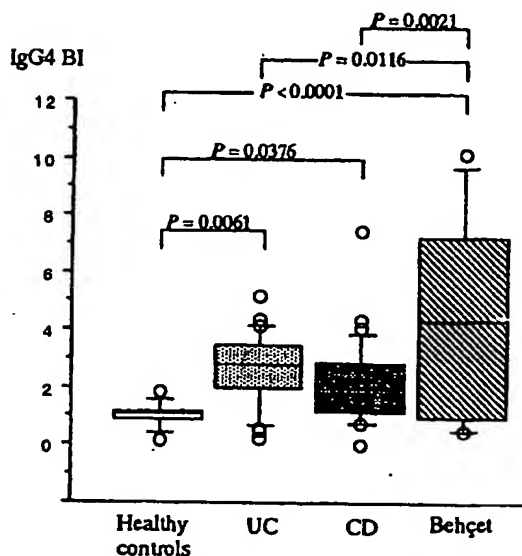


Figure 3 Binding index (BI) of IgG4 ASCA. IgG4 BI was significantly increased in patients with UC and CD compared with healthy controls, and it was significantly increased in patients with intestinal Behçet's disease compared with healthy controls, UC and CD.

with celiac disease [6,7,17]. We compared ASCA titers of patients with intestinal Behçet's disease as a disease control. In our study, differential elevation of IgG subclasses were found in patients with IBD and intestinal Behçet's disease. Interestingly, only IgG4 ASCA was significantly elevated in patients with IBD. IgG1, IgG3 and IgG4 ASCA were significantly elevated in patients with intestinal Behçet's disease.

IgG ASCA has been shown to be elevated in CD patients with small bowel involvement [7,9]. Meanwhile, Ruemmele *et al.* [17] showed that patients with CD limited to the colon tended to have lower IgG ASCA compared with others with CD, but did not reach significant differences. Such discrepancies could be explained by differential reactivities against strains of *S. cerevisiae* [7,16,17]. In this study, we found elevation of IgG3 ASCA in CD patients with small bowel involvement compared with the patients with only large bowel involvement.

Differential biological properties of IgG subclasses have been reviewed [11]. IgG1 and IgG3 had strong complement binding activity but IgG2 and IgG4 did not (or poorly) fix complement. IgG3 had a shorter half life and higher fractional catabolic rate than other subclasses. Chronic inflammatory states are often associated with serum IgG4 elevation [11]. Elevation of serum IgG1/IgG2 ratio in patients with UC compared with CD may reflect autoimmune disorder rather than response to infectious or stimulatory agents in patients with UC [18,19]. IgG1 subclass was the predominant fraction of IgG reactive against tropomyosins, which was spontaneously produced by lamina propria mononuclear cells from UC mucosa [20]. On the other hand, IgG4 antibodies were most frequently detected in sera from patients with UC in subclass studies of anticolon antibody [21]. IgG1 and IgG3 were the predominant subclasses of p-ANCA in patients with UC [22,23], in contrast to relatively high concentrations of IgG4 subclass in vasculitis-associated ANCAs [23]. IgG1 antiendothelial antibody levels were increased, but IgG2 and IgG4 antiendothelial antibody were decreased in CD compared to UC [24]. Seibold *et al.* [25] reported that IgG1 and IgG2 were predominant in subtype I, and IgG2 was predominant in subtype II anti-pancreatic antibodies in CD. So-called Th1 CD4 + T cells induced IgG2 and IgG3 production, while Th2 CD4 + T cells helped produce IgG1 class antibody, and altered Th1/Th2 balance may lead to autoimmunity in various autoimmune experimental models [26].

In terms of serum antibodies reactive with dietary protein antigens, most of such antibodies included IgG4 subclass [27,28]. Elevation of IgG4 ASCA in patients with IBD may reflect chronic immunological reactivity against dietary antigens or suggests chronic stimulation by luminal or colonic epithelial antigens [21]. IgG1, IgG3 and IgG4 elevation of ASCA in intestinal Behçet's disease may reflect rather complex autoimmune responses that result from chronic inflammatory states.

Favourable effects of a yeast-excluded diet in CD may indicate the importance of yeast in the pathogenesis of CD

[10]. Peripheral lymphocytes from healthy controls that included three bakers (highly exposed to baker's yeast) did not proliferate, but peripheral lymphocytes from patients with CD proliferated in response to baker's yeast implies participation of genetic factor in humoral response against *S. cerevisiae* [29]. They also found the absence of proliferation by yeast stimulation in CD patients receiving olsalazine maintenance therapy [29], which was consistent with our finding with IgG3 ASCA.

The etiological role of *S. cerevisiae* or ASCA remains unclear. Reduction of ASCA titers after intestinal resection in pediatric CD [17] suggests a humoral immune response to a luminal antigen across a disrupted mucosal barrier. However, *S. cerevisiae* alone could not be pathogenic but cross reactivity to various mannose-containing molecules may induce a hypersensitivity reaction [10].

In conclusion, elevation of IgG4 ASCA in patients with IBD represents the presence of chronic yeast stimulation in the pathophysiology of IBD, while IgG1, IgG3, and IgG4 elevation in patients with intestinal Behçet's disease demonstrates complex immunoresponses involved in this disease (Table 1).

Acknowledgements

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May 19, 22, 1991

LOW INTRAEPITHELIAL LYMPHOCYTES IN MALNUTRITION WITH GIARDIA OR STRONGYLOIDES STERCORALIS INFECTION

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Intraepithelial lymphocytes (IEL) and sIgA B cells are reduced in intestinal mucosa of children with protein-energy malnutrition (PEM). In Gabon, Central Africa, diarrhea is observed in all malnourished children infected by *Giardia intestinalis* or *Strongyloides stercoralis*, whereas asymptomatic carriage of those parasites is common in eutrophic children of the same age.

Intestinal mucosa biopsy was performed in 15 African children aged 11 to 34 months (mean 20.2) with PEM (mean weight 3.7 SD under standards) hospitalized for chronic diarrhea. *Giardia* trophozoites were found in duodenal juice of 5 infants, larvae of *Strongyloides stercoralis* in 4, and no parasites in 6, confirmed by stool examination. Severe mucosal atrophy was observed in all cases. IEL were always under the standards in normal European children or adults (9.5 to 40 IEL for 100 epithelial cells for *C. Anderson*). Means were 8.1% in *Giardia* infected children, 8.05% in *Strongyloides stercoralis*, and 7.9% in unparasitized and malnourished children. The rise of IEL after parasitic infections commonly observed in Europe, is not found in PEM. The pathogenicity of parasites more important in PEM is possibly in relation with the reduction of immunity in intestinal mucosa.

ANTIBODIES AGAINST *SACCHAROMYCES CEREVISIAE* (BAKER'S YEAST) IN CROHN'S DISEASE. H.H. Goffer, A. Clark, C.D. Holdsworth, Royal Hallamshire Hospital, Glossop Road, Sheffield S10.

A local intestinal immune response to an yet unidentified antigen(s) resulting in tissue damage and granuloma formation has been suggested as a possible mechanism of tissue injury in Crohn's disease. Both dietary and bacterial antigens have been implicated. It has been suggested that antibodies to *Saccharomyces cerevisiae* (baker's yeast) are specific to Crohn's disease and may play a significant role in the pathogenesis of this condition. To have therefore assessed the prevalence of IgG and IgA antibodies against *S. cerevisiae* in patients with inflammatory bowel disease. Forty nine patients with Crohn's disease, 43 with ulcerative colitis, 14 with coeliac disease and 20 healthy controls were studied. Coated cardia samples were tested for IgG and IgA antibodies to three *S. cerevisiae* strains (KVC 77, KVC 79 and KVC 1108) using a competitive ELISA technique. The antibody responses against all three yeast strains were similar. IgG titres were significantly raised in patients with Crohn's disease, (mean 0.51 \pm SEM 0.05) and those with coeliac disease (0.55 \pm 0.09) both levels being significantly different from those in ulcerative colitis (0.23 \pm 0.04), $P < 0.0001$ and healthy controls (0.18 \pm 0.03), $P < 0.0001$. The significant increase in IgA levels was, however, confined to patients with Crohn's disease. Disease location influenced IgG responses for patients with small bowel disease have significantly higher antibody titres than those with colonic disease. Although a difference in IgA levels also existed between small and large bowel disease, this was not statistically significant. Age, sex, disease activity, previous intestinal resection or treatment with elemental diet did not affect the IgG and IgA levels. Two patients with Crohn's disease who were clinically intolerant to yeast had high IgA but not IgG antibodies. It therefore concludes that IgG yeast antibodies are non-specifically raised in patients with Crohn's disease. IgA isotypes, however, were only increased in Crohn's disease. Their pathogenic significance remains unknown.

INTERLEUKIN-1B (IL-1B) ACTIVITY IN PATIENTS WITH ULCERATIVE COLITIS (UC). P. Bianchini, A. Compiani, A. Bolluzi, M. Forrotti, P. Bonio, C. Brignolo, P. Iannone, M. Miglioli, L. Borbora, Ist. Clinico Medico e Gastroenterologia, Osp. Controlisato, Policlinico S. Orsola, Bologna-Italy.

IL-1, a cytokine produced by macrophages and other cells, has a major role in inflammatory and immunological responses. Increased levels of IL-1 activity have been reported in experimental colitis and in patients with active Crohn's disease (CD) and UC. IL-1B release from fresh and cultured colonic biopsies and IL-1B serum concentrations were determined in 19 pts with active UC, 14 with UC in remission and 10 normal control subjects. Biopsies taken at colonoscopy were weighed, washed in ice of 9% sodium chloride solution and then cultured for 24 hrs in 10% fetal calf serum (FCS). IL-1B activity was determined by ELISA RIT (Cytrom Biotechnology) in plasma samples, in the washing solution and in the incubation medium. Very low levels of IL-1B were detected only in 3 plasma samples, all from active patients. Significantly more IL-1B was released from fresh and cultured colonic mucosa obtained from patients with UC in remission, compared with normal mucosa ($P < 0.01$). Furthermore, specimens from active UC released significantly more IL-1B than those from patients in remission ($P < 0.01$).

IL-1B RELEASE (pmol/1000 cells)

	Fresh tissue	Cultured tissue
Controls (10)	37 (18)	66 (41)
Active UC (19)	814 (276)	1240 (619)
Inactive UC (14)	92 (39)	420 (186)

In conclusion, IL-1B activity is only rarely found in plasma, even in active UC; colonic mucosal IL-1B release is significantly increased in patients with UC, correlated with disease activity, and may play an important role in mediating the inflammatory response.

MACROPHAGE SUBSETS IN NORMAL AND INFLAMED MUCOSA OF ILEAL POUCH ANAL ANASTOMOSIS (IPAA). P. Bianchini, G.H. Paganelli, A. Compiani, A. Bolluzi, C. Brignolo, P. Iannone, G. Bisceglia, G. Paggiolo, C. Gossotti, M. Miglioli, L. Borbora, Ist. Clin. Medico e Gastroenterologia, Clin. Chirurgica II, Univ. di Bologna-Italy.

Pouchitis is a well recognized complication of IPAA confined to patients operated on for ulcerative colitis (UC). Its cause is unknown. In this study we characterized macrophage subsets in ileal pouch biopsies from 8 patients with pouchitis (P) and 11 with normal pouch (NP). Tissue was snap-frozen and sections were obtained with monoclonal antibodies AFD1 (dendritic cells), AFD7 (mature macrophages), AFD9 (epithelioid cells and tingible body macrophages), using an immunoperoxidase technique. For each monoclonal antibody, positive macrophages as a percentage of the total number of mononuclear cells in the superficial lamina propria were determined by using a grid and counting, in blind, in three different areas of each section. There were no significant differences between P and NP in the number of positive macrophages for AFD1 and AFD7 antibodies, while there was a significant increase in AFD9 + cells in P (mean percentage (SD) [9.7 (3.0)] when compared to NP [0.63 (0.6)] ($P < 0.001$).

This histological pattern has been previously observed in UC and Crohn's disease. Its significance is unknown, but being AFD9 + macrophages not expressed in infectious colitis, their increase in P seems to suggest a different pathogenic mechanism to that of the original UC.

review

Hematologica 1995; 80:252-267

LACTOFERRIN: A GENERAL REVIEW

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ABSTRACT

Lactoferrin is a 703-amino acid glycoprotein originally isolated from milk. Plasma lactoferrin is predominantly neutrophil derived but indications are that it may also be produced by other cells. Lactoferrin in body fluids is found in the iron-free form, the monoferric form and in the diferric form. Three isoforms of lactoferrin have been isolated, ie two with RNase activity (lactoferrin- β and lactoferrin- γ) and one without RNase activity (lactoferrin- α). Receptors for lactoferrin can be found on intestinal tissue, monocytes/macrophages, neutrophils, lymphocytes, platelets, and on certain bacteria. A wide spectrum of functions are ascribed to lactoferrin. These range from a role in the control of iron availability to immune modulation. More research is necessary however to obtain clarity with regard to the exact mechanism of action of lactoferrin.

Key words: lactoferrin, lactotransferrin, iron-binding protein, immunomodulation

The name lactoferrin is derived from its past classification as a major iron-binding protein in milk. Lactoferrin, also referred to as lactotransferrin, was first identified in 1939 in bovine milk,¹ and in 1960 it was isolated from human milk by Johansson.² Subsequently it has also been shown to be a major iron-binding protein of other exocrine secretions such as bile, pancreatic juice and small intestinal secretions, and has been localized in a host of other tissues, both in man and in other mammals.³ The size and structure of lactoferrin is closely related to that of another group of iron-binding proteins, the transferrins, and lactoferrin is considered by many to be a member of the transferrin family.⁴ Plasma lactoferrin is currently considered to be predominantly neutrophil derived but indications are that it may also be produced by other cells. In the past it was traditionally seen as a mere bacteriostatic iron-transporting protein of milk, but this view is being challenged by recent research findings.

Structure and properties

The controversies surrounding lactoferrin

function are probably the result of misconceptions and ignorance about its structure. The complete amino acid sequence of human lactoferrin has been determined and found to contain 703-amino acid residues.⁴ Hololactoferrin consists of a single polypeptide chain folded into two globular lobes, each with one iron binding site.⁵ Iron binding to lactoferrin occurs concomitantly with the bonding of two bicarbonate anions, a process essential for the ligation of iron to lactoferrin.⁶ There is a notable degree of internal homology between the two lobes (residues 1-338 and 339-703, respectively), which demonstrates 125 (or 37%) identical amino acid residues in the corresponding portions.⁴ This has led to a theory of gene duplication, proposed to have occurred some 500 million years ago when the original 40 kDa molecule duplicated, forming the two domains and thus giving rise to a family of proteins with molecular masses in the range of 80 kDa (Table 1).⁷ Lactoferrin is suggested to be the youngest of the transferrins.

Lactoferrin is a basic glycoprotein with an isoelectric point of 8.7.^{8,9} Human milk lactoferrin has two poly-N-acetyl-lactosaminic glycans

Table 1. Molecular masses of lactoferrin by various methods

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Milk (apo form)
Milk (apo form)
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Table 1. Molecular weight of human milk lactoferrin as determined using various methods.

	Molecular weight (daltons)	References
Milk (apo form; by electrophoresis)	76,800	(5)
Milk (apo form; by sedimentation)	75,000	(5)
Milk (apo form; theoretical)	76,400	(5)
Milk (from amino acid sequence)	82,400	(4)
Milk (dry weight determination)	78,000	(18)
Milk (holo form; by sedimentation)	82,600	(7)

that contain N-acetylneuraminic acid (sialic acid), fucose and galactose.⁴² These sugars have been found to bind to asparagine residues 137 and 478, one located in the C- and the other in the N-terminal zone.⁴³ The primary structure of human polymorphonuclear neutrophil (PMN) lactoferrin glycans is identical to that of the major glycans from human milk lactoferrin. The two glycans, attached to lactoferrin through N-glycoside linkages, are nonetheless structurally heterogeneous and differ from those of other transferrins.⁴⁴ The precise role of these glycans has not been established, and their removal is said to have no apparent effect on lactoferrin functions and properties, such as receptor binding.⁴⁵ However, this assumption has been contested by isolated studies in which a role in receptor binding was implicated.¹¹

Lactoferrin is remarkably resistant to proteolytic degradation by trypsin and trypsin-like enzymes, rendering it at least partially resistant to digestion in the gut.⁶ This property, postulated to be glycan-dependent, facilitates neonatal absorption of lactoferrin from maternal milk. It is of interest that the iron-saturated form (ie hololactoferrin) is more resistant to proteolysis than the apoform.¹² Lactoferrin not only binds iron but copper, zinc, manganese, gallium,^{12,13} and possibly vanadium as well.¹⁴ The degree of lactoferrin iron saturation in plasma is unknown.¹⁵

Similarities between lactoferrin and other transferrins, like transferrin and ovotransferrin, are pronounced. The same polypeptide folding pattern is found in all members of the transfer-

rin family.¹⁶ Lactoferrin, like transferrin, is an iron transporter and as such exists in both the hololactoferrin (iron-saturated) and apolactoferrin (iron-depleted) form. The molecular mass of transferrin (apo-form: 75-76.6 kDa; holo-form: 73.8-86 kDa) lies within the reported range for lactoferrin (apo-form: 75-76.4 kDa; holo-form 82.6 kDa).⁵ The amino acid compositions of lactoferrin and transferrin were found to be closely related,^{11,17} with 59% and 49% homology between the two corresponding domains of the respective molecules.⁴ The secondary structures, including their disulfide linkages,¹⁸ as well as the tertiary ones⁹ are notably similar. These findings have led to speculation that the two molecules may share the same phylogenetic origin.¹¹ Lactoferrin, however, differs from transferrin in its immunologic or antigenic properties, carbohydrate composition, water solubility, isoelectric point, and the localization of its iron binding and glycosylation sites.^{11,17,20} Lactoferrin and transferrin have, as previously mentioned, comparable molecular masses with similar C-terminal and N-terminal iron-binding domains, consisting of β -sheets as well as α -helices.⁹ The inter-lobe connecting peptide is however helical in lactoferrin, while in transferrin it is irregular. The binding site for each lobe, which houses the Fe^{3+} and CO_3^{2-} ions, lies deep within the inter-domain cleft. The iron binding sites in the N- and C-lobes are similar: three anionic ligands, 2 tyrosine and 1 aspartic amino acids, with a fourth neutral histidine amino acid that matches the plus three charge on the metal ion, forming a hydrogen bonding network.⁹ The role of the carbonate anion is proposed to be twofold: (a) it neutralizes positive charges which might otherwise repel the cation, and (b) it partially prepares the metal binding site on the apo-protein by adding two more potential ligands.⁹

Crystallographic studies have shown conformational changes upon iron-binding in both lactoferrin and transferrin.⁹ Iron-binding affinities and characteristics of the individual lobes have been well studied for transferrin,²¹ but less is known about these characteristics for lactoferrin. Transferrin can exist in any of four molecular forms:^{11,22} apotransferrin, monoferric

transferrin, either in the A- or B-form, and diferric transferrin. As the degree of iron saturation increases the apparent molecular mass of transferrin decreases, implying that as iron binds to transferrin the binding areas must induce a conformational change that leads to a closed iron-binding domain. Separation of three lactoferrin forms has also been successfully performed using high-performance liquid chromatography, but absolute certainty about the existence of four iron-binding forms of lactoferrin has not as yet been achieved, since differentiation between possible A- and B-forms of monoferric lactoferrin by electrophoresis has not been carried out.²⁴

It has long been recognized that apotransferrin and iron-saturated transferrin differ in their reactivities to specific antisera on crossed immuno-electrophoresis.²⁵ These findings have severe implications for determinations in which the antibodies used were raised against only one of the forms of the transferrins. The conformational change in lactoferrin that occurs when it binds iron, and its implication in lactoferrin level determination is also emphasized by findings that hololactoferrin has an altered plant lectin binding capability with respect to the apoform.²⁶ This gives additional substance to findings that certain forms of lactoferrin have a higher affinity for lactoferrin receptors than others.¹⁹ The specific receptor affinity of lactoferrin and transferrin could perhaps also be ascribed to the difference between lactoferrin and transferrin inter-domain interactions.⁹ The molecule exhibits a pronounced tendency to polymerize *in vitro* as well as *in vivo* at concentrations as low as 10^{-10} M.^{27,28} This may possibly further contribute to the wide range of reported serum lactoferrin levels.

Lactoferrin is known to exist in various isoforms.¹ Three such isoforms, two with RNase activity (termed lactoferrin- β and lactoferrin- γ) and one without RNase activity (termed lactoferrin- α), have been isolated; all three are present in both human breast milk and in granulocytes.^{4,29} These isoforms share the same physical, chemical and antigenic characteristics, but differ in their functional properties. The iron-independent isoforms with RNase activity do not

exhibit functional iron-binding, while the iron-binding isoform has no RNase activity.⁴ These findings may partially explain the reported diversity in functions attributed to lactoferrin.

Lactoferrin levels in plasma

Lactoferrin is present in plasma in relatively low concentrations, with substantially higher levels being found in colostrum, human breast milk, and seminal plasma. Markedly higher levels occur in cord blood, tears, and vaginal mucus (Tables 2-4). The reported differences are probably attributable to factors such as (a) analytical methods, (b) the type of anticoagulant used, (c) variations in lactoferrin iron saturation, (d) the reported spontaneous *in vivo* as well as *in vitro* polymerization,^{27,28} and (e) the time interval between venipuncture and analysis or storage.

Plasma lactoferrin is predominantly neutrophil derived.⁴ Its presence in specific granules is often used to identify these granules. However, recent findings have shown that lactoferrin is also found in other granules, probably tertiary ones, albeit in lower concentrations.³⁰ Plasma lactoferrin concentrations may or may not correlate with the neutrophil count,³¹⁻³³ depending on the magnitude of degranulation and perhaps the contribution of other organs, such as bone marrow, endometrium and placenta, to the plasma content of lactoferrin.³⁴⁻³⁶ A summary of other lactoferrin-containing tissues has been provided elsewhere.¹⁹

Several authors reported higher lactoferrin levels in males than in females;^{17,27-29} one reported similar levels, but a greater standard deviation for females,¹⁷ and yet another reported higher levels in females than males.⁴⁰ In view of the higher granulocyte lactoferrin content found in men by Freeman *et al.*,⁴¹ one cannot dismiss the higher plasma levels in males reported by the majority of workers as a mere degranulation difference.

Lactoferrin plasma levels change during pregnancy. The changes in maternal plasma lactoferrin levels manifest as a progressive rise in concentration, with stabilization at week 29 of pregnancy.¹⁹ Several factors may contribute to this:

Table 2.1

Blood

Blood

Uterus
Vagina
Cervix
Fetus

Placenta
Cord

Saliva
Tears

Sweat
Urine

Stool
Semen

Milk
Mucus

Table 2. Reported human blood lactoferrin levels.

Blood	Level	MD	MC	Ref. #
Blood	0.2-1.5 µg/mL	RIA	EDTA	(49)
	0.05-0.250 µg/mL	LSA	EDTA	(50)
	0.02-0.20 µg/mL	ELISA	—	(51)
	<1.00-3.50 µg/mL	RIE	Serum	(52)
	0.13-0.42 µg/mL	RIA	EDTA	(31)
	0.385±0.153 µg/mL	RIA	Serum	(33)
	1.520±0.560 µg/mL	RIA	Heparin	(53)
	0.292±0.110 µg/mL	RIA	Serum	(54)
	0.108±0.059 µg/mL	RIA	EDTA	(54)
	0.888±0.334 µg/mL	RIA	EDTA	(55)
	1.500±1.400 µg/mL	RIA	EDTA	(56)
	0.040-0.100 µg/mL	ELISA	EDTA	(57)
	0.134±0.079 µg/mL	ELISA	EDTA	(58)
	0.307±0.066 µg/mL	ELISA	Serum	(59)
	0.012±0.002 µg/mL	RIA	EDTA	(60)
	0.250-0.750 µg/mL	RIA	EDTA	(61)
	0.540±0.260 µg/mL	ELISA	Serum	(62)
	0.046-0.257 µg/mL	ELISA	EDTA	(63)
	0.168±0.100 µg/mL	ELISA	EDTA	(34)
	0.237±0.155 µg/mL	ELISA	Serum	(34)
♂	0.0978 µg/mL	ELISA	EDTA	(37)
	0.150±0.067 µg/mL	RIA	EDTA	(38)
	0.307±0.141 µg/mL	ELISA	EDTA	(40)
	0.206±0.060 µg/mL	RIA	EDTA	(39)
	1.620±0.077 µg/mL	RIA	EDTA	(17)
♀	0.0847 µg/mL	ELISA	EDTA	(37)
	0.100±0.048 µg/mL	RIA	EDTA	(38)
	0.326±0.127 µg/mL	ELISA	EDTA	(40)
	0.140±0.060 µg/mL	RIA	EDTA	(39)
	0.750±0.036 µg/mL	RIA	EDTA	(17)
(pre-menopausal)	1.74±0.10 µg/mL	RIA	EDTA	(17)
Venous plasma	0.122±0.040 µg/mL	EIA	EDTA	(34)
Capillary plasma	0.107±0.073 µg/mL	EIA	EDTA	(34)
Fetal serum	0.05 µg/mL	RIA	—	(36)
Cord blood (capillary)	25.8 & 28.0 µg/mL	RIA	EDTA	(17)
	0.385±0.113 µg/mL	RIA	EDTA	(34)
	0.02-0.30 µg/mL	ELISA	—	(51)
Infant (capillary)	0.267±0.176 µg/mL	RIA	EDTA	(34)
	0.269±0.163 µg/mL	RIA	EDTA	(34)
	0.176±0.165 µg/mL	RIA	EDTA	(34)

MD=method of determination; MC=method of collection; RIA=radioimmunoassay; ELISA=enzyme-linked immunosorbent assay; LSA=latex agglutination; EIA=enzyme immunoassay; EDTA=ethylenediaminetetraacetic acid; EIA=enzyme immunoassay; ELISA=enzyme-linked immunosorbent assay

Table 3. Reported lactoferrin levels in human neutrophils.

Neutrophils	Level	MD	Ref. #
Blood	15 µg/10 ⁶ neutrophils	—	(64)
	2.12 µg/10 ⁶ neutrophils	ELISA	(37)
	29.2±2.2 µg/10 ⁶ neutrophils	IRA	(41)
	1.78 µg/10 ⁶ neutrophils	ELISA	(37)
	20.4±2.0 µg/10 ⁶ neutrophils	IRA	(41)
	89.0±7.3 µg/mg cell protein	RE/FA	(65)
	59.6±5.5 µg/10 ⁶ neutrophils	IRA	(66)
	Term neonates ♂	12.0±0.6 µg/10 ⁶ neutrophils	IRA (41)
	Term neonates ♀	12.6±0.4 µg/10 ⁶ neutrophils	IRA (41)
	Neonates	30.6±6.1 µg/10 ⁶ neutrophils	IRA (66)
Newborn	43.2±7.0 µg/mg cell protein	RE/FA	(65)

MD=method of determination; RIA=radioimmunoassay; ELISA=enzyme-linked immunosorbent assay; RE/FA=rocket electrophoresis and fluorescent assay.

ua-derived lactoferrin³⁵ and, perhaps, (d) a hormonal influence on lactoferrin production by tissues other than the endometrium or decidua, such as breast acini.⁴⁴ Indications are that lactoferrin levels may indeed be influenced by endocrine activity. Such a lactoferrin-hormonal link is implicated by (a) the larger standard deviation seen in female serum,¹⁷ (b) the suggested endometrial lactoferrin production during the secretory phase of the menstrual cycle,³⁵ (c) the increase in plasma levels during pregnancy,³⁴ (d) in an indirect way, by the correlation between neutrophil count and urinary estradiol levels,⁴⁵ (e) the higher postmenstrual than premenstrual levels in vaginal mucus,⁴⁶ (f) the decrease in maximal vaginal lactoferrin levels found in women on oral contraceptives,⁴⁷ (g) the reported tendency of plasma lactoferrin levels to vary with the menstrual cycle,⁴⁸ (h) the differences between male and female levels,³⁷ (i) the hormonal dependency of prostate lactoferrin concentration,⁴⁹ and (j) the higher postmenopausal plasma levels.^{17,34} These findings may however be epiphenomenal in nature.

Lactoferrin levels in milk, amniotic fluid and neonates

Lactoferrin is present in the milk of all mammalian species investigated to date with the exception of the dog and the rat.⁵⁰ Levels in bovine milk are, however, significantly lower

(d) pregnancy-associated leukocytosis;⁴⁴ (b) the reported selective increase in the lactoferrin granular content of neutrophils, while myeloperoxidase content remains the same;⁴⁴ (c) a contribution to maternal plasma levels by decid-

than those in human milk. Masson et al.⁴⁰ in fact showed that the levels in human breast milk were the highest among the ten different species investigated. Approximately 30% of the iron in human milk is bound to lactoferrin.⁴⁰ It is estimated that only 6-8% of milk lactoferrin is iron saturated, which correlates with the finding of Makino and Nishimura⁴¹ that 95% of milk lactoferrin is in the monoferric and/or apolactoferrin form. Lactoferrin levels in human milk do not appear to be dependent on body iron status, but rather on the general state of maternal nourishment. Lactoferrin is said to be generally lower in malnourished mothers.⁴⁰

Various authors have found colostrum lactoferrin concentration to be significantly higher than that of milk ejected after this period. The levels in milk do not however decline any further upon prolonged lactation.⁷⁰ Although no correlation could be shown between gestational age and lactoferrin levels,⁷¹ there is general consensus that the colostrum of preterm deliveries contains significantly higher lactoferrin concentrations than that of full-term deliveries.^{71,72} It is unlikely that this could be attributed to either the relatively smaller volume or the higher neutrophil count in preterm colostrum, since there is no difference in protein levels between preterm and full-term colostrum and the difference in neutrophil count is too small to be responsible.^{72,73} The initially increased lactoferrin level in preterm colostrum then declines over the colostrum producing period.⁷⁴ The finding of markedly higher lactoferrin level in colostrum than in serum despite the substantially lower neutrophil count of colostrum suggests either the production of lactoferrin by mammary tissue, or the active transport of lactoferrin against a concentration gradient.

Lactoferrin levels in amniotic fluid were found to be undetectable before the 20th week of pregnancy.⁷⁴ A significant increase is said to occur around week 30, whereafter it remains high until term. Lactoferrin levels in the decidua, amnion and chorion membranes, trophoblast and umbilical cord are shown in Table 4. Indications are that amniotic fluid lactoferrin may be of decidual origin.⁷⁴ It is of interest that maternal plasma lactoferrin levels demonstrate a corre-

Table 4. Reported lactoferrin levels in various human secretions and tissues.

Fluid and tissue	Level	MD	Ref.#
Colostrum milk	5-7 mg/ml		(67)
(preterm)	6.76±1.50 mg/ml	RIA	(71)
(full-term)	3.10±0.50 mg/ml	RIA	(71)
	6.7±0.7 mg/ml	RIE	(70)
Transitional milk	3.7±0.1 mg/ml	RIE	(70)
Mature breast milk	1-2 mg/ml	RIE	(74)
(human)	1.97-3.20 mg/ml	RIA	(73)
	2.6±0.4 mg/ml	RIE	(70)
Amniotic fluid	2-32 µg/ml	RIA	(36)
Decidua	9-95 µg/g protein	RIA	(36)
Amniotic membrane	2-37 µg/g protein	RIA	(36)
Chorion membrane	2-26 µg/g protein	RIA	(36)
Trophoblast	5-35 µg/g protein	RIA	(36)
Umbilical cord	< 1 µg/g protein	RIA	(36)
Bronchial mucus	35.2±6.5 µg/ml	—	(75)
Tear fluid	2.2 mg/ml	ELISA	(76)
Vaginal mucus			
Postmenstrual	62.9-218 µg/mg protein	—	(46)
Premenstrual	3.8-11.4 µg/mg protein	—	(46)
♀ on oral contraceptives	≤ 19.8 µg/mg protein	—	(46)
Seminal plasma	1.18±0.74 mg/ml	RID	(77)
Synovial fluid	46.4±35.9 µg/ml	—	(78)

(MD) = method of determination; RIA = radioimmunoassay; RID = radial immunodiffusion; ELISA = enzyme-linked immunosorbent assay; RIE = rocket immunoelectrophoresis.

sponding initial increase and finally stabilize at week 29 of pregnancy.⁷⁴ It is therefore plausible that this increase in plasma lactoferrin during pregnancy could be of decidual origin. Amniotic lactoferrin concentrations are, surprisingly, the highest reported levels after those of colostrum, milk, tears and seminal plasma.

Lactoferrin production in the fetus depends on gestational age and was found, by immunohistochemical detection, from 13 weeks onwards.⁷⁵ The presence of lactoferrin in fetal salivary glands at a certain level of cytodifferentiation, and the reported decline in salivary gland lactoferrin shortly after birth suggest a contribution to fetal lactoferrin levels by organs other

than blood cells.¹⁷ It is also possible that some of the fetal lactoferrin may originate from amniotic fluid, which has significantly higher lactoferrin levels than either fetal or maternal sera. At present it does not appear that lactoferrin can cross the placenta.¹⁸ This view is supported by the demonstrated lack of correlation between maternal and neonatal lactoferrin concentrations.¹¹

Plasma lactoferrin levels in the neonate are still controversial. The first reported levels in cord blood were 25.8 and 28.0 $\mu\text{g/mL}$ ($n=2$), which were at least ten times higher than the values found in adults in the same study.¹⁷ Some authors detected differences of a less significant magnitude between full-term infants ($0.385 \pm 0.113 \mu\text{g/mL}$) and adults ($0.122 \pm 0.040 \mu\text{g/mL}$), while others could show no difference between neonatal and adult levels.^{11,19} Some even reported an inability to detect any lactoferrin in cord blood.²⁰

Independent from whether or not plasma lactoferrin levels in the neonate and infant are indeed elevated, these values would appear to stabilize at normal adult levels by the age of 15 weeks.¹¹ Neonatal plasma lactoferrin levels depend on various factors such as the neutrophil count, neutrophil lactoferrin content, degranulation characteristics, lactoferrin half-life, as well as possible maternal contributions to the fetal plasma lactoferrin pool. Neonatal leukocytosis, which disappears within a week after birth, is well known;²¹ however, some controversy exists with regard to granular lactoferrin content, which many report to be decreased,^{11,13,22} but which one group of workers found to be comparatively normal.²³

The general impression with regard to fetal lactoferrin release characteristics would appear to be that of a slight suppression of degranulation, possibly resulting from a subnormal ligand-receptor interaction.^{24,25} These findings would, to a degree, correlate with other reports of suboptimal leukocyte activity in the newborn.²⁶ By the same token less than normal RES activity may prolong lactoferrin half-life. The relatively high neonatal lactoferrin levels with respect to adult values cannot, however, be explained solely by an immature RES.

Immunogenicity of lactoferrin from different human sources

The question arises whether any difference exists between lactoferrin from various sources (eg. milk or neutrophils). A study employing double immunodiffusion analysis on human breast milk, colostrum, apolactoferrin and neutrophil lactoferrin did not reveal any obvious disparity among them.²⁴ The complete DNA sequence of the human mammary lactoferrin gene shows 99.7% agreement with a partial sequence of neutrophil cDNA, and a deduced amino acid homology of 97% to the sequence of human milk lactoferrin.⁴ Certain investigators, on the other hand, found a difference in the terminal fucose residues of its glycan chains (which are required for lactoferrin binding to macrophages) between neutrophil- and human milk-derived lactoferrin,²⁵ while others demonstrated that individual antibodies can be produced which could differentiate between milk and neutrophil lactoferrin.²⁴ However, it is possible that this observation could be the result of antibody specificity for various iron-saturation forms of lactoferrin. The majority of the existing studies were performed without considering the presence of various isoforms or the degree of lactoferrin iron-saturation.

Sample collection procedure for optimal lactoferrin levels

Correct specimen collection for lactoferrin analyses is of paramount importance. Variations in collection techniques such as the use of heparin instead of EDTA collection tubes are known to give rise to unreliable results.²⁴ For reliable results it is suggested that (a) EDTA be used as anticoagulant, (b) minimum stasis be applied during venipuncture, (c) separation/centrifugation be performed as soon as possible after blood sampling, or at the latest within 5 hours of blood collection, (d) if separation is delayed, blood be stored at 4°C, and (e) centrifugation be performed preferably at 4°C.

Metabolism of lactoferrin

Lactoferrin is produced in neutrophils and

stored, in the iron depleted state, in the specific granules and possibly in the tertiary granules.^{44b} It appears that the steroid-thyroid receptor superfamily works in concert to modulate lactoferrin gene expression. This supports the hypothesis that lactoferrin levels are hormone dependent. A detailed discussion is perhaps beyond the scope of this writing. Lactoferrin, unlike myeloperoxidase and some other granular products, is not synthesized as a larger precursor and was found to be unphosphorylated.⁴⁷ Lactoferrin transfer to its storage granules is dependent on acidification mechanisms and occurs through the medial and transcisternae of the Golgi apparatus.⁴⁷ It therefore appears to be processed like proteins destined for secretion. The neutrophil lactoferrin within these granules has two destinations: it can either be secreted into the surrounding tissues or blood,⁴⁸ or the granules can fuse with phagosomes.⁴⁴ Secretion from polymorphonuclear cells into the circulation is dependent on degranulation factors, which in turn appear to be dependent on the activation of guanylate cyclase, cGMP and protein kinase C (calcium dependent). This occurs in both aerobic and anaerobic conditions, is unaffected by the presence of hydrogen sulphide and is stimulated by interleukin-8 and surface bound IgG.^{44c} Plasma lactoferrin levels generally increase in iron overload, inflammation, infectious diseases, and during tumor development, demonstrating a multifactorial stimulatory mechanism for lactoferrin release from neutrophils.⁴⁹

Upon release lactoferrin binds metal ions, of which iron has been the most intensively studied. The precise relationship of serum apo- to hololactoferrin has not as yet been determined, because such determinations pose certain experimental difficulties. Lactoferrin removal from circulation appears to occur in one of two ways. First, lactoferrin can be removed from the circulation, as well as from the interstitial spaces, by what would appear to be receptor-mediated endocytosis into phagocytic cells such as macrophages, monocytes and other cells of the RES, with subsequent transfer of the iron to ferritin.^{53,54} In experiments conducted with rats, the half-life of injected hololactoferrin was pro-

longed threefold by blocking the RES.⁵⁴ Some controversy with regard to the cells involved in this manner of lactoferrin removal still exists.⁵⁵ The alternative way of lactoferrin removal would be its direct uptake by the liver through an iron saturation-independent, clathrin-dependent, calcium-dependent process of endocytosis.⁵⁶ Kupffer and liver endothelial cells, as well as hepatocytes appear to be involved.⁵⁷ The binding sites may perhaps be the same as those for transferrin binding, since lactoferrin was shown to inhibit transferrin uptake by rat hepatocytes.⁵⁸

Bennet and Kokocinski showed that labelled lactoferrin was rapidly cleared from the circulation by the liver and spleen, with all lactoferrin being removed within 7 hours after injection.⁵⁹ It is as yet not sure whether lactoferrin, like transferrin, is recycled.⁶⁰ Further research is needed to fully understand lactoferrin metabolism in the human adult.

The kidneys appear to play a role in lactoferrin clearance from the circulation since both lactoferrin and lactoferrin fragments were found in the urine of infants.⁶¹ It is of interest to note that the lactoferrin found in breast-fed infants is predominantly of maternal origin.⁶² Low molecular fragments of lactoferrin were also reported in stools.⁶³ Both fecal and urinary elimination of lactoferrin, however, need further investigation because significant controversy still exists.

Lactoferrin receptors

Lactoferrin is a basic protein with a high isoelectric point (8.7), enabling it to undergo nonspecific binding to many target cells or proteins.⁶ Some studies with lactoferrin fragments indicate that part of the N-lobe (residues 1-90) is involved in lactoferrin receptor binding.⁶⁴ Other studies however found regions in both the C- and N-lobes of human lactoferrin that bind to bacterial lactoferrin receptors.⁶⁵ Lactoferrin receptors have been identified in the gastrointestinal tract, on leukocytes and macrophages, platelets, and on bacteria. A summary of these receptors is presented in Table 5.

Recept

Intestin

Monocy

Macroj

Neutro

Platelet

Bacter

-Sple

-Adm

-Muc

-Muc

-Shi

Spleen

-Alb

-Gd

-Ca

-Ser

-Lys

-P

-Ch

Th

S

rol

ha

in

nu

th

up

La

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Receptors	Molecular mass (kDa)	Affinity constant (Ka)	Specificity	Ref.#
Intestinal	114 (nonreducing) 38 (reducing)	0.3×10^{-4}	+ hololactoferrin + apolactoferrin + deglycosylated lactoferrin + lactoferrin fragments - bovine lactoferrin - transferrin	(5,10)
Monocytes	-	4.5×10^{-4}	+ lactoferrin + transferrin	(99)
Macrophages	-	1.7×10^{-4}	+ lactoferrin	(91)
Neutrophils	-	2.2×10^{-4} 0.6×10^{-4}	+ lactoferrin	(100)
Platelets	-	13.6×10^{-4} 1.23×10^{-4}	+ lactoferrin + transferrin + bovine lactoferrin	(101)
Bacterial:	-	-	-	(102,103)
- <i>Staphylococcus aureus</i>	-	-	-	(74,104)
- <i>Aeromonas hydrophila</i>	-	-	-	(105,106)
- <i>Neisseria meningitidis</i>	-	-	-	
- <i>Breueria influenzae</i>	-	-	-	
- <i>Shigella flexneri</i>	-	-	-	
Silent:	-	-	-	(107,108)
- Albumin	-	-	-	(95,109)
- IGA	-	-	-	
- Casein	-	-	-	
- Secretory component	-	-	-	
- Lysosome	-	-	-	
- β -lactoglobulin	-	-	-	
- DNA	-	-	-	

Table 5. Lactoferrin receptors identified.

The biological role of lactoferrin

Some controversy still exists as to the exact role and mechanism of action of lactoferrin. It has now been shown that lactoferrin does indeed play a role in the host defense mechanism as well as in iron metabolism. Its role in the host defense mechanism involves much more than that of a mere bacteriostatic agent. Lactoferrin, in addition to its bacteriostatic function, can also exert a bactericidal effect and can curb the proliferation of other microbes such as fungi and viruses. Moreover, it has an extended role in the body's defense mechanism through its immune modulatory actions. The major role of lactoferrin in iron metabolism would appear to be in the control of iron availability. Other mechanisms in which lactoferrin is implicated include a growth regulatory function in normal cells, coagulation, and perhaps cellular adhesion modulation. Lactoferrin is known to have a tendency to bind to a number of other molecules or silent receptors. The functional significance is not clear but certain facts related to these interactions are slowly beginning to emerge.

Role in iron metabolism

Lactoferrin from maternal milk is known to be absorbed in the intact form from the gut of infants.²⁸ The observation of a higher lactoferrin concentration and a higher iron availability in human than in bovine milk gave rise to the hypothesis that lactoferrin might promote iron absorption in breast-fed infants. This appears to be substantiated by the finding of better iron absorption in breast-fed infants than in newborns on bovine milk-based formulas.¹⁰⁰ Whether lactoferrin does indeed augment iron absorption is, however, still controversial¹⁰¹ but several reports would seem to support such a possibility, among others:

- the ability of human enterocytes to extract iron from lactoferrin;
- the high lactoferrin uptake by enterocytes;
- the correlation of neonatal urinary iron excretion with milk lactoferrin content as well as with breast milk uptake;
- the transport of iron across the intestinal brush border by lactoferrin;
- the accumulation of iron from lactoferrin in brush border membrane vesicles.

Lactoferrin may perhaps affect cellular mechanisms through its influence on iron availability. Iron is known to affect a host of cell functions such as DNA, and to a lesser extent RNA and protein synthesis, the expression of lymphocyte surface markers, immunoglobulin secretion, interleukin-2 receptor expression and many others.¹¹¹ Lactoferrin could thus, through its effect on iron availability, indirectly influence a wide spectrum of physiological activities.

Host defense

The role of lactoferrin in the body's defense against micro-organisms is clinically manifested by the recurrent infections seen in patients with an absence of specific granules,¹¹² and by the altered granulocyte function associated with lactoferrin deficiency.¹¹³ This is experimentally confirmed by research results such as the protective effect shown by lactoferrin in experimental *E. coli* septicemia.¹¹⁴

The best known role of lactoferrin in the host defense mechanism is that of a bacteriostatic agent whereby the proliferation of bacteria is inhibited through its iron sequestering properties. Iron withholding as a defence against infection and neoplasia well described by Weinberg.^{7,115}

Lactoferrin is known to have a wide spectrum of microbiostatic activities. It is however fairly ineffective against those bacteria which are able to acquire their iron from either lactoferrin or transferrin.¹¹⁶ It is now known that lactoferrin, in addition to its bacteriostatic action, can also be bactericidal. The bactericidal effect of lactoferricin B, a peptide proteolytically derived from the N-terminal region of lactoferrin, is said to be several times greater than that of lactoferrin. Lactoferricin B was shown to be lethal to a wide spectrum of microbes, and to rapidly inhibit the colony-forming capability of most species tested.¹¹⁷ The bactericidal effect of lactoferrin is mediated by blistering, i.e. damaging of the outer bacterial membrane, with subsequent alteration of its permeability. The bactericidal membrane damage includes incorporation of lactoferrin into the membrane and subsequent dispersion of lipopolysaccharides (LPS) through

a cation (Ca^{++} , Mg^{++} or Fe^{+++}) modulated process.¹¹⁸ The lactoferrin/lactoferricin reactive component of the bacterial membrane is said to be a 38-kDa protein molecule, namely porin.¹¹⁹ Porin is normally shielded by the polysaccharide moiety of LPS, which reduces the anti-microbial effect of lactoferrin.

It would appear as if lactoferrin can also exhibit fungicidal and perhaps anti-viral effects. The exact antifungal effect is not yet clear, but it is known that monoproduct fractions of *Candida albicans* increases the number of fungi-phagocytosing polymorphonuclear cells and that lactoferrin cannot inhibit this growth in the absence of polymorphonuclear leukocytes.¹²⁰ Direct killing and suppression of the colony-forming capability of *Candida albicans* by the N-terminal lactoferrin fragment, lactoferricin B, has also been shown. The mechanism apparently corresponds to that involved in the lactoferricin killing of bacteria and is suppressed in the presence of Ca^{++} and Mg^{++} .¹²¹ Neutrophils were shown to have reduced lactoferrin content during viral infections. This acquired neutrophil lactoferrin deficit is suggested to be instrumental in superimposed postviral bacterial infections.¹²² The effect of lactoferrin on viral proliferation *per se* is still controversial.

Lactoferrin, as previously mentioned, appears to play an extended role in the host defense mechanism by modulating other immune processes. Observations that suggest such an immune modulatory role are presented in Table 6.

Lactoferrin and cellular proliferation

A number of studies suggest a role for lactoferrin in cellular proliferation. Such studies include better gastrointestinal development in newborn animals fed maternal milk as compared to newborn animals fed commercial formulas,^{104,105} increased thymidine incorporation with lactoferrin supplementation of milk formulas,¹⁴¹ and *in vitro* augmentation of thymidine incorporation into rat crypt cell DNA by lactoferrin.¹⁴² The dependence of lactoferrin growth stimulatory activity on iron saturation was shown by the fourfold higher DNA synthesis in a mouse embryo cell line under the influ-

Table 6. Host defense/immune modulatory function of lactoferrin.

Modulatory function	Probable mechanism	Ref. #
1. Lactoferrin enhances neutrophil accumulation at, and adherence to tissues of the site of injury	• Reduction in the surface charge and thus in the repulsive forces.	27
2. Lactoferrin enhances granulocyte "stickiness" and in so doing promotes cell-to-cell interaction	• Lactoferrin binds to the surface of polymorphonuclear cells and reduces the surface charge.	123
3. The controversy with regard to the role of lactoferrin in free radical production (ie inhibition or augmentation) probably depends on the environmental conditions which cause lactoferrin to be either an iron scavenger or an iron supplier:		
a) In acid environments such as in the phagolysosome, lactoferrin may promote the production of radicals for the intragranulocyte killing of microorganisms.	a) The furnishing of iron by lactoferrin to an oxygen radical-generating system.	124 65
(i) Lactoferrin catalyzes the neutrophilic production of hydroxyl radicals.	(i) Providing iron.	125
b) At normal extracellular pH values, lactoferrin may inhibit free radical production and in this way perhaps diminish oxidative damage to tissues.	b) Lactoferrin acts as an iron scavenger.	124,126
(i) It inhibits the production of free radicals by stimulated monocytes.	(i) Iron-binding dependent.	126
(ii) Lactoferrin may protect neutrophilic cells from lipid peroxidative damage.	(ii) Iron-binding dependent.	27
(iii) Lactoferrin inhibits lipid peroxidation mechanism.	(iii) Iron-binding dependent, since iron-saturated lactoferrin demonstrated no inhibitory effect	127
4. Lactoferrin, through its growth regulatory function, affects the host defense mechanism:		
a) The effects would appear to be predominantly inhibitory in nature.	(i) Unknown, but would appear to be dependent on its iron chelating properties.	128
(i) Lactoferrin inhibits mitogen- and alloantigen-induced human lymphocyte proliferation.	(ii) Unknown, but apparently iron saturation dependent.	129
(ii) Lactoferrin blocks histamine release from rat mast cells.	(iii) Unknown.	130
(iii) Lactoferrin inhibits the synthesis of antibodies.	(iv) Unknown.	131
(iv) Lactoferrin helps to control monocyte/macrophage activity.	(v) Controversial. Some found a procomplement action.	124
(v) Lactoferrin has an anticomplement action.	(vi) Unknown, but independent of lactoferrin iron saturation or lactoferrin RNase-activity.	83
(vi) Lactoferrin augments natural killer cell (NK) cytotoxicity and lymphokine activated killer cell (LAK) cytotoxicity.		
b) Most of the above mechanisms, shown to be affected by lactoferrin, are generally stimulated by cytokines. The mechanism of action of lactoferrin may thus be through its effect on cytokine activity. A couple of publications would appear to support this possibility.	(i) Concentration dependent lactoferrin inhibition of Interleukin-1 synthesis (negative feedback).	132,133
(i) Lactoferrin suppresses the secretion of granulocyte-monocyte colony stimulating factor.	(ii) Unknown.	133
(ii) Fifty percent iron-saturated lactoferrin inhibits the release of cytokines, such as tumor necrosis factor, interleukin-1 β and interleukin-2, in a dose- and time-dependent way.	(iii) Unknown.	133
(iii) Lactoferrin only affects the release but not the biological activity of the cytokines.	(iv) Not known, but independent of iron saturation.	126
(iv) Lactoferrin, in the presence of lipopolysaccharides, augments the production of interleukin-1 β , tumor necrosis factor- α , interleukin-6 and prostaglandins.		
5. Lactoferrin may modify the inflammatory response in SLE by binding to DNA	• Interaction between lactoferrin and DNA prevents the binding of anti-DNA. Lactoferrin is also able to disperse the anti-DNA-DNA bond.	134
6. Lactoferrin was shown to enhance the T-cell autoreactivity associated with Mycobacterium-induced arthritis.	• Cross reactivity between the mycobacterial 65-kDa heat shock protein and lactoferrin.	135
7. Neutrophil lactoferrin augments the antimicrobial capacity of macrophages.	• Macrophages ingest lactoferrin rich granulocytes as their source of lactoferrin and myeloperoxidase.	136
8. Lactoferrin enhances polymorphonuclear cell functioning by increasing their mobility and priming them to produce superoxide at a faster rate.	• Unknown; but apparently independent of iron saturation and can be abolished by anti-lactoferrin.	137
9. Lactoferrin potentiates the bactericidal capabilities of bactericectins, a class of arginine-rich antibacterial peptides of bovine neutrophil granules.	• The synergistic action of lactoferrin and bactericectins increases bacterial membrane permeability.	138
10. Lactoferrin is able to substitute for antibodies in order to activate the classical pathway of complement.	• Unknown, but involves the adherence of lactoferrin to the membrane.	124,139

ence of hololactoferrin than in the same line under the influence of apolactoferrin.¹⁴³ A role for lactoferrin as growth stimulatory factor in embryos and neonates is further suggested by the significant enhancement of DNA synthesis in rat neonatal hepatocytes by iron saturated lactoferrin. This mitogenic characteristic of lactoferrin apparently does not apply to adult rat hepatocytes.¹⁴⁴ The effect of lactoferrin on cancerous cells would appear to be inhibitory rather than stimulatory.¹⁴⁵ Some contradictions still exist however.

The exact effect of lactoferrin on myelopoiesis is still being debated. The contrasting views on this subject have previously been referred to as the *lactoferrin controversy*.² The reader is referred to a publication in which the controversial points of view are discussed.² The majority of research workers are presently of the opinion that lactoferrin acts as a negative feedback regulator of myelopoiesis.^{124,125,126,127} The mechanism of action would appear to be through suppression of the release of cytokines such as interleukin-1, tumor necrosis factor and interleukin-2.^{124,127} Lactoferrin has been shown to (a) bind to specific receptors on hemopoietic cells, (b) become internalized in such cells, and (c) associate with DNA within the nucleus.¹⁴⁶ Euchromatin has been suggested as the probable functional site for the lactoferrin inhibitory action.¹⁴⁶ Whether lactoferrin can directly influence hemopoietic cell proliferation, or whether its effect is primarily through the regulation of cytokine release must still be confirmed.

Other possible function

Acute phase proteins are defined as proteins whose concentrations in plasma increase by 25% or more following infection or inflammation.¹⁴⁷ Several authors have suggested that lactoferrin be classed as an acute phase protein.

An antithrombotic function has also been ascribed to lactoferrin. The possibility that lactoferrin or lactoferrin-derived substances may influence platelet function is supported by observations such as (a) the presence of lactoferrin receptors on platelet membranes,¹⁴⁸ (b) the inhibition of ADP-treated platelet aggrega-

tion,¹⁵⁰ (c) the inhibition of fibrinogen binding to ADP-treated platelets, and (d) the inhibition of platelet aggregation, thromboxane generation, serotonin release and α -granule membrane protein expression.¹⁵¹

In addition to its proposed role in the modulation of the host response, lactoferrin may also be involved in immunotolerance. It has been shown to prevent activation of the complement system (confirmed by hemolytic assay).¹⁵² Indications, however, also exist that it may activate the classical complement pathway.¹⁵³ The presence of anti-lactoferrin antibodies in certain autoimmune diseases might also imply a role for the molecule in immunotolerance (Table 7).

Possible clinical applications

Since lactoferrin is released in a nonspecific way in response to inflammation, any such event will increase its levels through neutrophil activation and degranulation. The diagnostic application of these levels is similar to that of several different indicators of immune stimulation, such as neopterin and elastase- α 1-proteinase inhibitor complex and others, rendering lactoferrin levels relatively nonspecific. A number of clinical applications are nonetheless described in the literature. These are mostly of diagnostic or prognostic predictive value and include plasma lacto-

Table 7. Diseases in which anti-lactoferrin antibodies have been shown to occur, and the frequency with which they occur.

Disease	Frequency or percentage of anti-lactoferrin antibodies	Ref.#
Crohn's disease	Occasionally, 34%, 8%	(152-154)
Ulcerative colitis	High, 45%, 50%	(152-154)
Primary sclerosing cholangitis	High, 50%	(152,154)
Uncomplicated RA	Occasionally, 10%, 2.4%, 4%, 20%	(152,155,156)
SLE	Occasionally, 20%, 15-20%, 39%	(152,155)
Primary Sjögren's syndrome	Occasionally	(152)
Scleroderma	19%	(155)
Felty's syndrome	50%	(156)

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ferrin determination as an index of the total blood neutrophil pool or neutrophil kinetics,^{11,13} as a tool in the diagnosis of chronic myeloid leukemia,¹⁴ granulocytic leukemia,¹⁵ chronic calcifying pancreatitis,^{16,17} cystic fibrosis,^{18,19} septicemia,^{20,21} congenital aplasia of the vasa deferentia and seminal vesicles,²² schizophrenia,²³ joint inflammation and cartilage degradation,²⁴ psoriasis²⁵ and rheumatoid arthritis.²⁶ Lactoferrin antibodies have been demonstrated in patients with Felty's syndrome, and the detection of these antibodies may prove useful in its diagnosis.^{27,28} It has further been suggested that β -lactoferrin/RNase and γ -lactoferrin/RNase may be of value in the detection of breast cancer.²⁹ The weak discriminatory power of changes in total plasma lactoferrin concentration makes it unlikely that the determination of values will ever achieve widespread prognostic or diagnostic application.

Conclusions

A wide spectrum of functions have been ascribed to lactoferrin. This may indicate a relative nonspecificity of function rather than a highly specialized role. It is possible that lactoferrin may exert most of its functions through its effect on iron availability, but this is difficult to explain in the light of our present knowledge about lactoferrin-iron affinity. More insight into the interrelationships and interactions between lactoferrin fragments, isoforms, and the different iron-saturated structures will no doubt go a long way toward providing a better understanding of the mechanism of action of lactoferrin.

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Correlation of Lactoferrin with Neutrophilic Inflammation in Body Fluids

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We have reported that lactoferrin, a 77-kDa iron-binding glycoprotein found in secondary neutrophil granules, provides a useful marker of fecal leukocytes in fecal specimens from patients with inflammatory diarrhea (R. L. Guerrant, V. Araujo, E. Soares, K. Kotloff, A. A. M. Lima, W. H. Cooper, and A. G. Lee, *J. Clin. Microbiol.* 30:1238-1242, 1992). In order to determine the usefulness of this marker of neutrophilic inflammation in different body fluids, we examined blood, gingival swabs, sputum, and saliva using antilactoferrin antibodies (lactoferrin latex agglutination [LFLA]). LFLA titers in whole blood samples were $\leq 1:4$ in all eight samples from patients with neutropenia (absolute neutrophil count [ANC] = <150 polymorphonuclear cells [PMNs] per μl), $\leq 1:8$ in samples from 13 individuals with moderate leukocyte counts (ANC = 150 to 8,000), and 1:8 to 1:32 in samples from six patients with neutrophilia (ANC $> 8,000$). While the overlap precludes a useful role in the identification of neutropenia, these data confirm that lactoferrin titers of $>1:100$ indeed indicate inflammation in fluid specimens. On quantitative elution of lactoferrin from gingival swabs, all 7 patients with dental plaque had titers of 1:200 to 1:400; 9 of 12 patients with clinical gingivitis had LFLA titers of 1:200 to 1:1,600, while all 7 individuals with healthy gums and teeth and 4 edentulous patients had LFLA titers of $\leq 1:100$. Eight purulent sputum samples had titers of $\geq 1:400$ (7 were 1:1,600) while 11 normal saliva samples showed titers of $\leq 1:100$. Lactoferrin titers in sputum, gingival swabs, and whole blood correlate with the presence of neutrophils or inflammation in these specimens and may offer a convenient rapid test for inflammatory processes.

In the process of neutrophil maturation, the first granules to appear are the azurophilic granules (or primary granules). These granules are lysosome-like and contain acid hydrolases, degradative enzymes, and defensins (antimicrobial cationic proteins). Following the primary granules, the secondary granules appear during the metamyelocyte stage of maturation and contain vitamin B₁₂-binding protein and lactoferrin (18).

Lactoferrin is a 77-kDa iron-binding glycoprotein that facilitates the production of hydroxyl radicals and chelates iron, preventing its accessibility to microorganisms and thus inhibiting their growth (18). Its structure is related to that of the serum iron transport protein transferrin, but only traces of lactoferrin are normally found in serum (14). It is present in several body secretions in various concentrations: 7 $\mu\text{g}/\mu\text{l}$ in colostrum, 1 $\mu\text{g}/\mu\text{l}$ in mature milk (15) (although a second increase occurs toward the end of lactation [10]), 4.7 to 26 ng/ μl in saliva (19), and 3.8 to 218 $\mu\text{g}/\text{mg}$ of protein in vaginal mucus (depending on the time in the menstrual cycle, being highest just after the menses [4]). Lactoferrin is specifically found in secondary granules in polymorphonuclear neutrophils (PMNs) and is not found in lymphocytes or monocytes (8, 12, 16). However, the lactoferrin found in neutrophil granules differs from that found in milk in that it lacks terminal fucose residues in the glycan chains that are required for binding to macrophages (5). The *in vitro* effect of lactoferrin may be bacteriostatic or even bactericidal for a wide range of microorganisms, including gram-positive and gram-negative bacte-

ria, aerobes, anaerobes, and yeasts (20). By sequestering iron, the free lactoferrin (apolactoferrin) blocks its utilization by bacteria, blocking microbial carbohydrate metabolism (2) or destabilizing the bacterial cell wall, perhaps through binding of calcium and magnesium (6). The bactericidal activity of lactoferrin may relate to its providing iron that can catalyze the production of free radicals, which lead to microbial killing within the phagolysosome of neutrophils (13). The antimicrobial role of neutrophil lactoferrin is further demonstrated in patients whose neutrophils lack specific granules and who, therefore, suffer from recurrent infections (3).

We have described the feasibility and potential cost effectiveness of the measurement of fecal lactoferrin as a marker of fecal leukocytes (9). Since World War I, when Wilmore and Shearman, and other physicians from the British service, investigated the use of fecal "cytodiagnosis" in the differential diagnosis of bacillary and amoebic dysentery, the use of microscopic examination of fecal smears with methylene blue staining has been proposed as a rapid test that may serve as a useful adjunct to other clinical features in the provisional diagnosis of inflammatory diarrhea (1, 7, 21). However, there are limitations to this examination. It requires the availability of a skilled microscopist and a microscope, and the microscopic exam must be done on a fresh cup (not swab) specimen because leukocyte morphology tends to be lost during storage and on swabs (9). Therefore, the simple *in vitro* test for the presence of lactoferrin in fecal specimens developed by our group has proved to be a useful procedure for detecting inflammatory diarrheas in nearly 90% of the cases studied (17). Furthermore, it was useful even with stored and swab specimens (9). It has also been used to identify inflammatory vaginitides (18a). The purpose of this study was to extend this work

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respectively. The LFLA assay may provide an easy screening test for the purulence of clinical specimens and may thus help determine when specimens warrant further microbiologic evaluation.

The number of PMNs found in sputum may vary considerably with the area examined in a fresh expectorated sputum specimen which should be collected for Gram staining and culture. While the LFLA test is highly sensitive to the numbers of PMNs seen, it does not exclude the contamination of the sputum by oropharyngeal secretions. Therefore, while the LFLA may be useful for detecting the presence of PMNs, positive specimens should then be examined microscopically for squamous epithelial cells as well as for predominant microorganisms (11). Decisions about culture and treatment, however, must be made in the clinic, and a quick, simple screening test for sputum purulence that does not require microscopy might be a very useful and practical tool to determine when further microscopic or microbiologic study is indicated.

While it does not exclude contamination from other sites (e.g., oral bacteria in a sputum sample), the LFLA assay provides a quick, simple means to detect the presence of PMNs that does not require sophisticated technical expertise or microscopic examination and could be done as a screening test possibly in a clinic or accession laboratory. One hundred percent of purulent sputum samples were positive at $\geq 1:400$ (87.5% had titers of 1:1,600), whereas 100% of control saliva had titers of $\leq 1:100$. Comparable results were obtained with gingival swab specimens, with 75% of 12 specimens having titers ranging from 1:200 to 1:1,600 in patients with clinical gingivitis while all samples from edentulous patients and normal controls had titers of $\leq 1:100$. Mild levels of inflammation were less clearly related to clinical status and were found in patients with dental plaque as well as in those with mild gingivitis.

In an era of growing need for applicable technologies for improved clinic-based diagnosis and selective therapy in developing areas and in improved cost containment in industrialized countries, the LFLA assay may hold considerable promise. Clearly, further studies are needed to define its clinical usefulness for patients being evaluated for possible gingivitis or respiratory tract infections. The application of this method by using dipstick technologies, on which we are currently working with colleagues at Techlab, would further extend its potential usefulness.

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Perinuclear Anti-Neutrophil Cytoplasmic Antibodies Are Spontaneously Produced by Mucosal B Cells of Ulcerative Colitis Patients¹

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Approximately 60% of sera from ulcerative colitis (UC) patients contains Igs reactive with neutrophil components, raising the question of the origin of these anti-neutrophil cytoplasmic Abs (ANCA). Our assertion that ANCA is a marker for a mucosal disease-related immune response predicts the existence of ANCA producing B cell clones in the lamina propria lymphocyte (LPL) fraction of UC patients. This hypothesis was tested by examining 12-day culture supernatants of LPL ANCA expression. LPL were isolated from surgically removed mucosa from patients with UC, Crohn's disease (CD), and diverticulitis. Normal mucosa was obtained from accident victims or normal margins of colon cancer resections. Supernatants were assayed by a fixed neutrophil ELISA. The ANCA staining pattern of supernatants expressing ANCA, as determined by ELISA, was assessed by indirect immunofluorescent staining of alcohol-fixed neutrophils. ANCA was found in 70% of culture supernatants from UC LPL fractions. In contrast, only approximately 11% of supernatants from CD and diverticulitis/normal (noninflammatory bowel disease (IBD)) LPL displayed ANCA binding. A perinuclear (pANCA) staining pattern was obtained with 70% of ANCA-expressing UC LPL supernatants, whereas ANCA-expressing CD and non-IBD LPL supernatants displayed a cytoplasmic reaction. PBL and mesenteric lymph node lymphocytes lacked spontaneous pANCA production, and pANCA production from PBL was not inducible. These findings indicate the existence of pANCA-producing B cell clones in mucosal lesions of UC patients and support our hypothesis that pANCA production is a consequence of a mucosal immune response specific to UC. *The Journal of Immunology*, 1995, 155: 3262-3267.

The last 5 years have seen tremendous progress in the study of anti-neutrophil cytoplasmic Abs (ANCA).³ These Abs have provided the foundation for new methods of disease identification, assessment of disease progression, and understanding the pathogenesis of a variety of autoimmune diseases (1-7). By using indirect immunofluorescence (IIF), ANCA expression has been divided into two broad categories: cytoplasmic neu-

trophil staining (cANCA) and a perinuclear to nuclear staining or cytoplasmic staining with perinuclear highlighting (pANCA). We and others have identified pANCA serum reactivity in 68% to 80% of ulcerative colitis (UC) patients (8-15). Titers of these pANCA do not correlate with disease activity, duration, or extent (8, 10).

The presence of ANCA in the serum of UC patients may well be a marker of an underlying selective immune regulatory abnormality manifested by a specific type of inflammation that may differ from inflammation in UC patients without this marker. This hypothesis was generated from the findings of several recent studies that have shown different familial, genetic, and clinical associations between UC patients expressing ANCA and those who do not (16-18). These findings support the thesis that ANCA is a marker that defines the mucosal inflammatory process of some UC patients. If ANCA, especially pANCA, is a marker for a disease-related immune response process, it is assumed that there are B cells within the colonic mucosa that produce these Abs.

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³ Abbreviations used in this paper: ANCA, antineutrophil cytoplasmic Abs; IIF, indirect immunofluorescence; cANCA, cytoplasmic antineutrophil cytoplasmic antibody; pANCA, perinuclear antineutrophil cytoplasmic antibody; UC, ulcerative colitis; LPL, lamina propria lymphocyte; MLN, mesenteric lymph node; MLNL, mesenteric lymph node lymphocyte; IBD, inflammatory bowel disease.

The purpose of this study was threefold: 1) to determine whether ANCA- and pANCA-producing B cell clones are present in UC mucosa, 2) to correlate the specificity of the presence of ANCA and pANCA for UC, and 3) to determine the site(s) of origin of "spontaneous" production of pANCA. Results demonstrated the presence of pANCA reactivity in the lamina propria lymphocyte (LPL) population of mucosa from UC patients, but not from the mucosa of Crohn's disease (CD), diverticulitis, or "normal" patients. Furthermore, the absence of a pANCA reaction in supernatants from the PBL and mesenteric lymph node lymphocyte (MLNL) fraction isolated from pANCA-expressing UC patients implicates the mucosa as the site of origin for production of these Abs.

Materials and Methods

Patient population

Intestinal tissue (from either the large or small bowel) was obtained from patients undergoing surgical resection. Samples were obtained from 33 patients with UC, 17 with CD, and 9 with diverticulitis. In some cases, multiple samples were obtained from the same patient. Diagnosis was based on at least two, and often all four, of the following criteria; clinical, radiologic, endoscopic, and histologic. All patients with inflammatory bowel disease (IBD) had undergone surgical resection because of intractability or complications of bowel disease, and many, but not all, were on steroid therapy at the time of surgery. The 10 normal (control) tissue samples were obtained from resections for colon carcinoma at least 10 cm from the areas of pathology and appeared histologically normal. PBL were obtained from an additional 25 patients with UC who had been evaluated and treated at the Cedars-Sinai IBD Center. Intestinal tissue (mucosa and mesenteric nodes) was obtained from six of these patients undergoing surgical resection because of complications of bowel disease. Institutional review board approval was obtained.

Cell isolation and culture

LPL from human intestinal mucosa were isolated as previously described by Bull and Bookman (19) and modified as described by MacDermott et al. (20–22). In brief, mucosa was dissected free of muscularis and washed in HEPES buffered calcium- and magnesium-free HBSS containing 5% human serum and antibiotics (wash buffer). After weighing and mincing, the 2- to 5-mm mucosal pieces were stirred with 0.75 mM EDTA containing wash buffer for 45 min at 37°C. This treatment was repeated until no more crypt cells were released in the wash solution. The mucosal pieces were digested by collagenase (16 U/ml, Worthington Biochemical, Freehold, NJ) in wash buffer supplemented with 10% human serum, with constant stirring at 37°C in a humidified atmosphere of 5% CO₂:95% air. LPL were separated from the digestion supernatant by Ficoll-Hypaque (Piscataway, NJ) gradient centrifugation as previously detailed (8).

In vitro Ab synthesis

To examine spontaneous Ig production, isolated LPL and PBL were washed extensively and cultured at 37°C in a humidified atmosphere of 5% CO₂:95% air for 12 days at a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS and antibiotics as described by MacDermott et al. (22). Supernatants from these cultures were analyzed for IgG content by solid-phase RIA as also detailed in MacDermott et al. (22). In preliminary experiments, some PBL and MLN specimens were examined for the inducibility of pANCA by the addition of anti-CD40 (1 µg/ml) and IL-4 (5 µg/ml) at the time of culture initiation.

ELISA

A fixed neutrophil ELISA was used to detect ANCA as previously described by Saxon et al. (8). All samples were analyzed in a blinded fashion. Briefly,

2.5×10^5 neutrophils were added to each well of a microtiter plate, allowed to settle, air-dried, and fixed with 100% methanol. After blocking nonspecific binding with 0.25% BSA in PBS, supernatants from cultured LPL, PBL, or MLN lymphocytes diluted 1:2 in blocking buffer were added. Neutrophil-bound Ab was detected by reaction with alkaline phosphatase-conjugated goat F(ab')₂ anti-human IgG γ -chain specific Ab (Jackson ImmunoResearch, West Grove, PA) and reduction of *p*-nitrophenyl phosphate (Amresco, Solon, OH). Color development as measured at 405 nm using a microplate reader (Molecular Devices, Menlo Park, CA).

Indirect immunofluorescence

The neutrophil binding pattern of ANCA in LPL, PBL, or MLN culture supernatants was determined in a blinded fashion by IIF as described by Saxon et al. (8). Slides prepared by cytocentrifugation of human neutrophils were fixed with 100% methanol and incubated with undiluted supernatant from cultured LPL. Bound Ab was detected by the binding of a fluorescein-conjugated goat F(ab')₂ anti-human IgG γ -chain specific Ab (Tago, Burlingame, CA), and the reaction was viewed under epifluorescent illumination.

Statistical analysis

Results are expressed as mean \pm SD except for the IgG concentrations which are given as the geometric means \times /+ SD. Statistical significance between groups was determined by two-tailed Student's *t*-test in which significance is defined as a *p*-value of <0.05 .

Results

LPL from UC mucosa spontaneously produce ANCA

ANCA found in serum of UC patients could be derived from either the systemic or the mucosal immune system. If we postulate that ANCA is a marker for a disease-related immune response, then ANCA-producing B cell clones should be found in the lamina propria. Since it has been shown that there is an increase in spontaneously secreted IgG in UC, we chose to look for ANCA-producing B cells using freshly isolated LPL cultured for 12 days without exogenous stimulation of B cell IgG production. Supernatants were collected from cultured LPL derived from colonic surgical specimens of disease-involved and uninvolved tissue from UC and CD patients, as well as patients with diverticulitis and normal mucosa. The latter two groups were termed "non-IBD patients." Supernatants were tested for the presence of ANCA at a 1:2 dilution by a fixed neutrophil ELISA developed in our laboratory (8). Results demonstrate that 70% (23/33) of all supernatants derived from UC LPL were ANCA reactive (Fig. 1 and Table I). In contrast, only approximately 11% of culture supernatants derived from both CD and non-IBD LPL expressed ANCA ($p < 0.02$ and $p < 0.01$, respectively, vs UC).

Majority of ANCA in supernatants from UC LPL have a perinuclear reaction pattern

IIF staining of alcohol-fixed neutrophils has shown that the majority of UC serum ANCA displays a perinuclear reaction pattern (8, 10, 14). To assess the reaction pattern

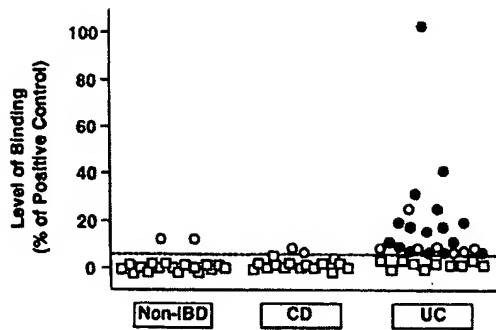


FIGURE 1. Levels of binding by supernatants from cultured LPL of normal subjects and patients with diverticulitis (non-IBD), CD, and UC in a fixed-neutrophil ELISA. Negative values (\square) are defined as those below the mean of normal values \pm two SD as represented by the dashed line. Positive samples are further distinguished by IIF as those binding in a cytoplasmic pattern (\circ) vs a perinuclear pattern (\bullet).

Table 1. Summary of alcohol-fixed neutrophil ELISA data and correlative IIF staining pattern of cultured LPL supernatants

Mucosal Type	n	ANCA Positive n (% of Samples)	IIF Pattern n (% of Positive Samples)		IgG Level $\mu\text{g/ml}$
			pANCA	cANCA	
UC	33	23 (70%) ^a	16 (70%)	7 (30%)	25 \times/\div 28 ^b
CD	17	2 (12%)	0 (0%)	2 (100%)	15 \times/\div 26 ^b
non-IBD ^c	19	2 (11%)	0 (0%)	2 (100%)	8 \times/\div 6 ^b

^a $p < 0.01$ vs non-IBD and $p < 0.02$ vs CD.

^b A subset of total samples were assayed for IgG level; $n = 22$ for UC, $n = 8$ for CD, $n = 15$ for non-IBD.

^c This group was comprised of 10 (histologically normal) mucosal samples obtained from resections for colon carcinoma and 9 mucosal samples from patients with diverticulitis.

of ANCA in supernatants from cultured LPL, undiluted supernatants from samples expressing ANCA by ELISA were exposed to methanol-fixed neutrophils. Seventy percent (16/23) of the ANCA generated by the LPL of UC patients displayed a perinuclear reaction pattern (Fig. 2, A and B). The 11% of ANCA-expressing supernatants from non-UC LPL all reacted with neutrophils with a cytoplasmic reaction pattern. Finally, in agreement with the ELISA assay, most of the non-UC and non-IBD LPL supernatants that did not express ANCA were also negative by IIF. A summary of the ELISA results and the ANCA IIF staining pattern correlations for all samples examined is given in Table I.

To assess whether spontaneous production of pANCA by LPL is a unique feature of B cells in that compartment, the 12-day culture supernatant of the PBL fractions of 25 UC patients seropositive for ANCA were assayed by both ELISA and IIF. Only four (4/25) PBL samples were found to spontaneously express ANCA, but only one (1/25) of those had a pANCA pattern as assessed by IIF staining using methanol-fixed neutrophils. Additionally, to determine if pANCA production by PBL could be stimulated,

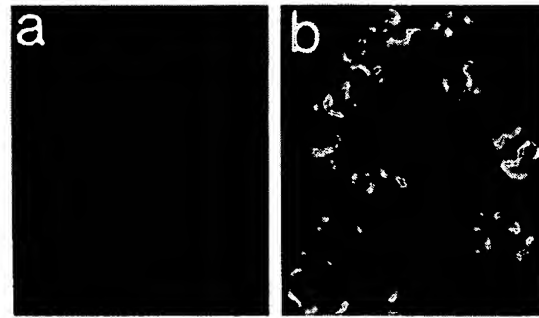


FIGURE 2. ANCA staining patterns of supernatants from cultured LPL determined by IIF staining of alcohol-fixed neutrophils. A, Negative reaction produced by an ELISA-negative supernatant from cultured LPL of a normal subject. Note that neutrophils are barely visible. B, A strong perinuclear (pANCA) staining pattern produced by the ELISA-positive supernatant of cultured LPL from an UC patient. This staining pattern is similar to that obtained with sera from patients with UC that are seropositive for ANCA.

19 of the UC PBL samples were cultured under in vitro conditions known to activate B cells and elicit IgG production (23, 24); i.e., in the presence of IL-4 (5 ng/ml) and anti-CD40 (1 $\mu\text{g/ml}$). Only two (2/19) PBL samples could be stimulated to produce pANCA. Finally, PWM (1:100) failed to stimulate any ANCA production in this PBL panel (data not shown).

To examine the correlation of ANCA production in the various B cell compartments, supernatants were generated from LPL and PBL, as well as from MLN cells from 6 of the 23 UC patients seropositive for ANCA and characterized with respect to serum ANCA staining pattern. Five of the six (5/6) serum ANCA were found to be pANCA, while one was a cANCA. All five of the LPL supernatants from pANCA seropositive patients expressed ANCA and displayed a pANCA staining pattern identical to that of its matched serum ANCA (Table II). The majority (4/6) of the PBL supernatants did not express ANCA, and those (2/6) that did express ANCA displayed a cANCA immunofluorescent reaction pattern (Table II). An examination of supernatants from cultured MLNL revealed that most of these cells did not spontaneously produce ANCA (Table II).

Total IgG levels do not correlate with ANCA expression

Since an increase in the spontaneous production of total IgG by LPL from UC patients was demonstrated (22), the presence of ANCA in UC supernatants could be due to this increase in IgG. Total IgG was measured in each LPL culture supernatant using RIA. As seen in Figure 3, no correlation was found between the level of total IgG in the LPL supernatants and the level of ANCA binding. Additionally, while incubation of PBL in the presence of IL-4

Table II. Correlation of spontaneous pANCA production in supernatants of LPL, PBL, and MLN with serum pANCA values from UC patients seropositive for ANCA by ELISA

Patient No.	Serum		LPL		PBL		MLN	
	Binding level (%)	IIF pattern	Binding level (%)	IIF pattern	Binding level (%)	IIF pattern	Binding level (%)	IIF pattern
1	91 (+) ^a	P ^b	11 (+)	P	5 (-) ^c	n/a	3 (-)	n/a
2	61 (+)	P	19 (+)	P	3 (-)	n/a	5 (-)	n/a
3	24 (+)	P	19 (+)	P	5 (-)	n/a	0 (-)	n/a
4	17 (+)	C	1 (-)	n/a	15 (+)	C	3 (-)	n/a
5	156 (+)	P	15 (+)	C/P	12 (+)	C	7 (+)	P
6	93 (+)	P	103 (+)	P	4 (-)	n/a	3 (-)	n/a

^a (+), ELISA (ANCA)-positive samples defined as binding in fixed neutrophil ELISA in excess of two SD above mean of normal control samples.

^b P, Perinuclear immunofluorescence pattern with alcohol-fixed neutrophils; C, cytoplasmic immunofluorescence pattern with alcohol-fixed neutrophils; n/a, not applicable in ELISA-negative samples.

^c (-), ELISA-negative samples defined as binding in fixed neutrophil ELISA less than two SD above mean of normal controls. For nonserum samples (LPL, PBL, MLN) LPL supernatants of normal LPL was used as normal control sample, while serum from normal donors served as normal control for patient serum.

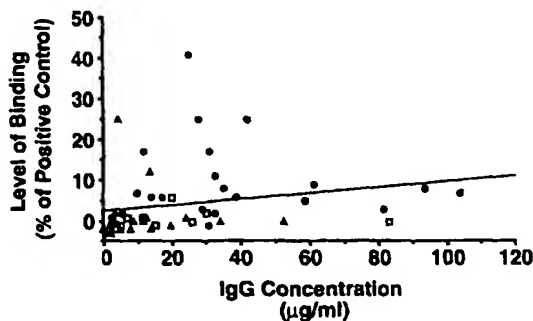


FIGURE 3. Correlation of binding level in a fixed neutrophil ELISA to IgG concentration in supernatants from cultured LPL of non-IBD (normal subjects and patients with diverticulitis) (Δ , $r = 0.00$), CD (\square , $r = 0.08$), and UC (\bullet , $r = 0.00$). The solid line indicates linear regression of combined points ($r = 0.20$).

and anti-CD40 resulted in the stimulation of IgG production for most of the samples examined (11/13), with some attaining IgG levels similar to those of the LPL supernatants, there was no correlation between enhancement of IgG level and ANCA production (data not shown). It can be concluded that the increased level of LPL ANCA and pANCA reactivity is likely to reflect a specific disease-related immune response.

Discussion

This study demonstrates the presence of ANCA-secreting B cells within the mucosal LPL fraction from 70% of patients with UC. IIF analysis showed that the majority of these ANCA (70%) are pANCA (Table I). In contrast, only approximately 11% of CD and non-IBD LPL produced ANCA after 12 days of culture. None of these displayed a pANCA reaction pattern (Table I). Of significance is the finding that spontaneously pANCA-secreting B cells seemed not to be present in the majority (24/25) of the PBL fractions nor could these cells be stimulated to produce ANCA using a combination of stimuli known to

augment IgG1 production (24). Also, the ANCA staining pattern produced by the LPL supernatant correlated well with that of the corresponding serum (Table II). The finding of ANCA positivity is not simply due to enhanced levels of IgG in UC LPL supernatants (Fig. 3). Rather, their presence suggests a disease-specific relationship. Indeed, the absence of antitetanus toxoid titers in most of the LPL supernatants (data not shown) supports the contention that Ab production by B cells in this compartment is not merely a consequence of the nonspecific activation of B cells by a cytokine-rich environment such as is found in the inflamed mucosa. Two recent studies support our contention that the intestinal mucosa may be a unique source of autoantibody-producing B cells. One study showed that mononuclear cells extracted from the colon but not from the peripheral blood, spleen, or draining lymph nodes of a UC patient with severe autoimmune hemolytic anemia were able to spontaneously produce Ig when cultured *in vitro*. Additionally, only the colon-derived mononuclear cells produced IgG with anti-red cell activity, albeit only when placed in SCID mice (25). A second report (26) showed that oral administration of LPS to antierythrocyte autoantibody transgenic mice resulted in the activation of peritoneal and LPL B-1 cells even in animals that congenitally lacked lymph nodes and Peyer's patches (*aly* mice), suggesting that the cells in these two compartments form a common pool independent of Peyer's patches and lymph nodes. This latter result indicates that B cells may be directly activated in the mucosal compartment by enteric Ags, and suggests that the presence of Ab in serum is due to spill over of that production.

Finally, in a preliminary experiment, cultured lymphocytes isolated from the lymph nodes of two UC patients exhibited no spontaneous production of pANCA (Table II) but could be stimulated to produce pANCA by incubation with IL-4 and anti-CD40 (data not shown), suggesting the presence of primed but not previously activated autoimmune B cells in this compartment.

The ability to detect pANCA presence in UC LPL supernatants indicates either a high proportion of pANCA-producing B cell clones in the lamina propria of UC patients or a high production of pANCA by a small number of B cells because B cells account for only about 20% of the LPL fraction. Further studies will be required to determine whether auxiliary cells within the LPL fraction are required for B cell secretion of pANCA. However, in preliminary experiments, CD19⁺ cells isolated from LPL of two UC patients were also capable of producing pANCA following 12 days of culture. Although previous results have indicated an increase in "spontaneous" IgG production by LPL from UC patients (22), no disease-related Ag-specific responses have yet been identified for this IgG fraction. The finding of ANCA production, especially pANCA production, by the LPL fraction of mucosa from UC patients is the first Ag-specific B cell response found in UC.

Recent work has shown a direct pathogenic role for the ANCA of Wegener's granulomatosis and systemic vasculitis (27–32). When these Abs bind to the neutrophil surface, they trigger an array of responses, which culminate in target cell destruction (30, 31). By contrast, the pANCA generated in UC do not seem to recognize intact (unpermealized) neutrophil surfaces and no alterations in neutrophil function due to UC ANCA binding have been found (Vidrich et al., unpublished observations). Titers of these ANCA do not correlate with disease activity or duration (8, 10), and the role that these locally produced Abs play in the pathogenic process is not yet known. However, a number of immune-mediated diseases, such as SLE, primary biliary cirrhosis, and chronic active hepatitis are closely associated with distinctive patterns of autoantibodies (33–35). Although these autoantibodies are probably not directly pathogenic, they are markers for their particular disease and serve as useful tools for the diagnostic and genetic evaluation of disease and may have an integral place in the immunobiology of the autoimmune response.

The data presented in this study indicate that pANCA are being produced by local B cell populations within the lamina propria and are limited to mucosal tissues from UC patients. Further, the preliminary data demonstrating the induction of pANCA from B cells within MLNs suggests that these B cells are being primed in the afferent compartments of the mucosal immune system. It is well known that although titers drop, pANCA persist in the serum of UC patients following colectomy (8, 10, 11, 36, 37). One possible explanation for this persistence is that pANCA production is related to and indicative of an underlying immune dysregulation, but a more intriguing possibility is that lamina propria cells from other parts of the mucosa, in particular the small intestine, may well be capable of producing pANCA in patients with UC. Although no direct proof is currently available, support for this hypothesis comes from recent studies showing that almost 50% of patients who undergo colectomy and receive an ileal pouch-anal anastomosis eventually develop pouchitis (36,

38–40). In addition, 88% to 100% of patients who have pouchitis express pANCA compared with only 18% to 50% of patients who do not develop pouchitis (38). These studies suggest that pANCA-expressing B cells may reside in the mucosa of small bowel. Under appropriate conditions that result in chronic inflammation, such as are found in ileal pouches (i.e., bacterial overgrowth and/or fecal stasis), these B cells are triggered to express pANCA. Although it is clear from the current study that lamina propria cells from UC patients spontaneously produce pANCA, it does not rule out the possibility, as suggested by the above-mentioned studies, that other parts of the intestine can produce this Ab response and, in conjunction with additional factors, give rise to expression of disease.

B cells can function as APCs for T cell activation *in vivo* (41). This pivotal immunologic role may be fundamental to abridgment of tolerance to self-antigen and in the activation of pathogenic T cells. Since preferential Ag presentation by B cells reflects the exceptional avidity of membrane Ig for specific Ag, then Abs produced by at least a subgroup of B cells in autoimmune lesions may actually identify the target Ag of the pathogenic T cell without direct involvement in the disease pathogenesis (41). Understanding pANCA-secreting B cells within the mucosa of UC patients and their role in induction of and the nature of the response of mucosal T cells will give important insight into the mechanism of this disease specific inflammatory process and its role in the altered mucosal inflammation of ANCA-expressing UC patients.

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Utility of a Rapid Fecal Latex Agglutination Test Detecting the Neutrophil Protein, Lactoferrin, for Diagnosing Inflammatory Causes of Chronic Diarrhea

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Objective: The utility of tests for fecal neutrophils in the setting of chronic diarrhea has not been established. The purpose of this study was to determine the causes of chronic diarrhea associated with fecal neutrophils. **Methods:** One fecal specimen from each of 10 normal subjects, 26 patients with known microscopic colitis, 13 with celiac sprue, eight with Crohn's disease, four with ulcerative colitis, and 103 with chronic diarrhea of unknown origin, as well as 10 fecal specimens from a patient with chronic nongranulomatous enterocolitis were analyzed blindly for the presence of a neutrophil granule protein called lactoferrin using a commercial latex agglutination kit. Diagnostic evaluation of the 103 patients with chronic diarrhea was carried out to determine the diagnostic accuracy of this test for chronic inflammatory bowel disease. **Results:** None of the normal control subjects, three of 39 patients with microscopic colitis or celiac sprue, all 10 specimens from the patient with enterocolitis, and all 12 control patients with ulcerative colitis or Crohn's disease had a positive fecal lactoferrin test. Eleven of 103 patients with chronic diarrhea presenting without a diagnosis had a positive test, and all were diagnosed with an inflammatory condition of the colon (five-, ulcerative colitis; four-, Crohn's disease; one-, ischemic colitis; and one-, microscopic colitis). Only one patient with inflammatory bowel disease had a negative lactoferrin test. The sensitivity, specificity, and positive and negative predictive values of the fecal lactoferrin test for ulcerative or Crohn's colitis were 90%, 98%, 82%, and 99%, respectively. **Conclusion:** The major cause of fecal neutrophils in patients with chronic diarrhea is chronic inflammatory bowel disease of the colon. The latex agglutination test for fecal lactoferrin offers a highly sensitive, specific, and simple means for detection of fecal neutrophils in these patients. (Am J Gastroenterol 1998;93:1300-1305. © 1998 by Am. Coll. of Gastroenterology)

INTRODUCTION

Evaluation of patients presenting with chronic diarrhea is challenging to both generalists and gastroenterologists because of the multitude of diseases that cause this symptom. For this reason, it would be desirable if the number of potential etiologies could be limited by noninvasive, inexpensive screening tests performed at the outset of diagnostic investigation. Analytical tests of stool may accomplish this objective. One such test involves an analysis of feces for the presence of leukocytes; detection of fecal neutrophils in a patient with chronic diarrhea (as in the setting of acute diarrhea) provides evidence that intestinal inflammation, likely involving the colon, is present.

Staining and microscopy is the traditional method of detecting white blood cells in fecal excretions (1, 2). However, the utility of this method is limited by several factors. These include: the necessity that specimens be refrigerated and analyzed within 24 to 48 h before the leukocytes degenerate (3, 4); the inability to differentiate confidently neutrophils from other leukocytes or other cells (5); physician uncertainty of local medical technologist skill and experience evaluating fecal specimens; and confusion regarding the significance of vaguely reported results (such as "few WBCs seen"). In the last several years, a rapid latex agglutination method that immunologically detects a glycoprotein constituent of neutrophilic granules, lactoferrin, has been developed. The test is highly sensitive for detecting fecal leukocytes (*i.e.*, it is able to detect the fecal neutrophil microscopy equivalent of one cell per high power field), but does not detect monocytes or lymphocytes because these cells do not contain lactoferrin (3). This assay for lactoferrin, available commercially as Leuko-Test (from Tech-Lab, Blacksburg, VA), circumvents the aforementioned drawbacks with fecal neutrophil microscopy. It is advantageous in that it has been shown to be more sensitive and specific than microscopy for detecting neutrophilic inflammation in feces (3, 6-10); it does not require rapid analysis of specimens because breakdown of leukocyte cell structure does not affect test results (3); negative and positive results can

be differentiated with little subjective bias; and it has proven valuable in the diagnosis of acute inflammatory diarrhea caused by bacillary dysentery or *Clostridium difficile*-induced pseudomembranous colitis (6–9, 11–13).

The utility of tests for fecal leukocytes in the setting of acute diarrhea has been studied widely (1, 2, 14–22). However, in patients with chronic diarrhea, the diagnostic implications of a positive or negative test for fecal neutrophils has not been clearly established. Therefore, in this study, we prospectively tested stools from 103 consecutive patients with chronic diarrhea of undetermined etiology, 47 patients with known inflammatory bowel diseases of various histopathologic types, and 10 normal control subjects for the presence of neutrophils. Because of the apparent advantages and simplicity of the latex agglutination test, and because stools were to be collected over 48–72 h, we chose to use Leuko-Test rather than microscopy. We hypothesized that 1) stools from patients with active inflammatory bowel diseases that usually involve significant neutrophilic inflammation of the intestine, such as Crohn's disease and ulcerative colitis, would be positive for fecal lactoferrin, and therefore, that the Leuko-Test would be sensitive for these diagnoses in patients with chronic diarrhea; and 2) stools from patients with noninflammatory diarrheal conditions or with diseases that involve mainly mononuclear inflammation, such as celiac sprue and microscopic colitis, would be negative for lactoferrin (meaning that a positive Leuko-Test would be specific for ulcerative colitis or Crohn's disease). If this hypothesis is correct, the detection of lactoferrin in stools of patients with chronic diarrhea may improve the time- and cost-effectiveness of diagnostic evaluation by leading to a direct pursuit of inflammatory bowel disease.

METHODS

Control samples

Negative controls. Stools from 10 healthy volunteers (five men and five women), a normal control group, and stools from 26 patients with untreated microscopic colitis and 13 with untreated celiac sprue, hypothetically a negative disease control group, were tested in duplicate for the presence of lactoferrin.

Positive controls. Eight patients with active untreated Crohn's disease (six with disease involving the colon and small bowel, two thought to involve only the small bowel), and four patients with ulcerative colitis (two newly diagnosed, and two refractory to medical treatment) were tested in duplicate for the presence of lactoferrin. Ten fecal specimens collected over a one-month period from a patient with refractory non-granulomatous enterocolitis also were tested for lactoferrin.

The technicians performing these analyses were unaware of the diagnoses of any of the patients or subjects in the negative and positive control groups.

Patient samples

Patients with chronic diarrhea of unknown etiology. Stools from 103 consecutive patients presenting to Baylor University Medical Center over a 6-month period (February through August, 1997) for evaluation of chronic diarrhea of unknown etiology were tested prospectively for the presence of lactoferrin. All patients had completed a 48- or 72-h quantitative fecal collection that was weighed and analyzed for its electrolyte and fat content. These data were used to guide further diagnostic investigation by their physicians that ultimately led to a final diagnosis. However, the physicians conducting these investigations were unaware of the Leuko-Test results. Patients underwent extensive evaluation (including endoscopic biopsies of the colon and small intestine, quantitative culture of small intestinal contents, small bowel radiography, and, when appropriate, assay of various hormones in blood and/or urine, and a CT scan of the abdomen) before their diarrhea was ascribed to the "idiopathic" variety.

Two hundred gram aliquots from the stool collections were frozen for possible future retesting. Frozen stools from patients ultimately discovered to have inflammatory bowel disease were thawed at study's end and reanalyzed for the presence of lactoferrin to assess the reproducibility of the Leuko-Test and the stability of lactoferrin under these storage conditions.

Method of fecal lactoferrin detection

The commercial **Leuko-Test kit** (TechLab, Blacksburg, VA) was used to detect lactoferrin in stools as follows: 50 μ l of stool was placed on a test card and diluted 50-fold with 2.5 ml of a buffered protein solution containing 0.1% sodium azide (supplied with the kit). One drop of a solution containing rabbit-IgG antibody sensitized to human lactoferrin and attached to latex beads was then added. In the presence of lactoferrin, the beads agglutinate and appear as white particulate matter visible on the black background of the test card (a positive test). Each specimen was analyzed in the presence of a positive and negative control provided with the kit. The test takes less than 5 minutes to complete and materials cost approximately \$5 per test.

Analysis of fecal occult blood

Several studies of acute infectious dysentery have touted the benefits and simplicity of a guaiac card test detecting hemoglobin as a surrogate marker of intestinal inflammation (9, 19–22). Therefore, to explore this issue in the setting of chronic diarrhea, fecal specimens from the 103 patients presenting without a diagnosis were tested for the presence of occult blood with Hemoccult II (SmithKline Diagnostics, San Jose, CA) in standard fashion.

TABLE 1
Results of Fecal Lactoferrin Testing in 103 Patients With Chronic Diarrhea and Their Final Diagnoses

Diagnoses in 11 Patients With a Positive Fecal Lactoferrin Test	
Diagnosis	No. of Patients
Ulcerative colitis	5
Crohn's disease of small bowel and colon	4
Ischemic colitis	1
Microscopic colitis	1
Diagnoses in 92 Patients With a Negative Fecal Lactoferrin Test	
Diagnosis	No. of Patients
Secretory diarrhea*	46
Malabsorption†	29
Microscopic colitis	8
Celiac sprue	5
Microscopic colitis and celiac sprue	3
Ulcerative colitis‡	1

* Characterized by a high concentration of electrolytes in stool without steatorrhea; causes of diarrhea mainly were idiopathic but also laxative abuse, irritable bowel syndrome, postcholecystectomy, diabetes, and fecal incontinence. Pathophysiologically some of these etiologies are not secretory processes, *per se*.

† From pancreatic disease, non-inflammatory short bowel syndrome, postgastrectomy, hyperthyroidism, and cholestasis.

‡ Upon repeat testing, the fecal specimen from this patient was positive for lactoferrin.

RESULTS

Fecal lactoferrin in normal subjects and patients with known causes of chronic diarrhea

All of the fecal specimens from the normal subjects were negative for lactoferrin. Only two of 26 patients with microscopic colitis (8%), and one of 13 patients with celiac sprue (8%) had a positive Leuko-Test. In contrast, all 12 patients with known Crohn's disease or ulcerative colitis had lactoferrin detected in stools, as did all 10 fecal specimens from the patient with nongranulomatous enterocolitis. Duplicate testing displayed 100% concordance with initial results.

Fecal lactoferrin in patients with chronic diarrhea of unknown cause and their final diagnoses

Eleven of the 103 patients with chronic diarrhea had a positive fecal lactoferrin test on initial testing of fresh stool and later repeat testing of thawed frozen stool. All of these patients were ultimately discovered to have an inflammatory disease of the colon: five had newly diagnosed ulcerative colitis, four had active Crohn's disease involving both small bowel and colon (three newly diagnosed, one established), one had ischemic colitis involving the right and transverse colon, and one had microscopic colitis (Table 1).

Of the 92 patients with chronic diarrhea of unknown origin and a negative fecal lactoferrin test, only one was diagnosed with active inflammatory bowel disease (ulcerative colitis; see Table 1). The stool specimen from this

TABLE 2
Sensitivity, Specificity, and Predictive Value of the Fecal Lactoferrin Test for Ulcerative Colitis and Crohn's Disease in Patients With Chronic Diarrhea

Sensitivity	9 true positives ÷ 10 with disease = 90%
Specificity	91 true negatives ÷ 93 without disease = 98%*
Positive predictive value	9 true positives ÷ 11 total positives = 82%*
Negative predictive value	91 true negatives ÷ 92 total negatives = 99%

* 100% for any inflammatory disease of the colon (including one case of ischemic colitis and one of microscopic colitis).

patient, which had been stored frozen following initial analysis (as all specimens had), was thawed after 2 months of storage and retested with the Leuko-Test on two separate occasions (2 wk apart). On repeat analysis, the test was positive both times. The remaining 91 patients with a negative fecal lactoferrin test were discovered to have a variety of diagnoses. Most of these diagnoses were noninflammatory; 16 had celiac sprue, microscopic colitis, or both (Table 1).

From these results, the sensitivity, specificity, and positive and negative predictive values of the Leuko-Test for active ulcerative colitis or Crohn's disease as a cause of chronic diarrhea were calculated and are shown in Table 2. Although the positive tests in the two patients with ischemic colitis or microscopic colitis probably represent true positives in terms of the presence of fecal neutrophils, for the purposes of the calculations in Table 2, they are considered false positives (because they are not the inflammatory diseases hypothesized to be detected by Leuko-Test; see Introduction).

Results of fecal Hemoccult testing and correlation with fecal lactoferrin

Table 3 shows the results of testing for fecal occult blood by Hemoccult II and its correlation with results of the Leuko-Test in patients with the major causes of chronic diarrhea identified. Occult blood was detected in seven of the 11 patients (64%) with inflammatory bowel disease accompanied by neutrophilic inflammation (*i.e.*, ulcerative colitis, Crohn's disease, or ischemic colitis). Twenty-two of the 92 patients with mononuclear inflammatory diseases or noninflammatory diagnoses (24%) had Hemoccult-positive stools.

DISCUSSION

In this study, we hypothesized that a latex agglutination test that immunologically detects the neutrophil granule protein lactoferrin in stool would be sensitive and specific for causes of chronic diarrhea associated with neutrophilic inflammation, mainly Crohn's disease and ulcerative colitis. This hypothesis was substantiated, as 17 of 18 patients either known or found to have these latter diseases had lactoferrin detected in stool. Although one patient each with

TABLE 3
Correlation of Fecal Leuko-Test and Hemocult II in the Major Causes of Inflammatory and Noninflammatory Chronic Diarrhea Identified

Disease	n	Leuko-Test		Hemocult II	
		(+)	(-)	(+)	(-)
Neutrophilic inflammatory diseases					
Ulcerative colitis	6	5	1	4	2
Crohn's disease	4	4	0	2	2
Ischemic colitis	1	1	0	1	0
Total	11	10	1	7	4
Mononuclear inflammatory diseases					
Celiac sprue*	8	0	8	3	5
Microscopic colitis	9	1	8	0	9
Total	17	1	16	3	14
Noninflammatory diseases					
Secretory diarrhea	46	0	46	9	37
Malabsorption	29	0	29	10	19
Total	75	0	75	19	56

* Three of these patients also had microscopic colitis.

ischemic colitis and microscopic colitis also had a positive fecal lactoferrin test, no patient without intestinal inflammation tested positive. These results were obtained under the most ideal of analytical circumstances; the technicians performing the lactoferrin tests were unaware of the patients' diagnoses and the physicians making the diagnoses were unaware of the results of the lactoferrin test.

A second aspect of our hypothesis was that chronic diarrheal conditions that strike mainly a mononuclear cell reaction in intestinal tissue or that are not associated with any form of inflammation would not be associated with fecal lactoferrin. For the most part, this also was true as no patient with a noninflammatory condition had a positive test, and only four of 55 patients with microscopic colitis or celiac sprue (7%) had lactoferrin detected in stool. (It is known that some of these patients manifest a mild mucosal neutrophilic inflammatory response) (23, 24). Although these results are consistent with the mainly plasmacytic and lymphocytic inflammatory reaction of these diseases, they are in contrast to one study that reported a 55% prevalence of "fecal leukocytes" by microscopy in 116 patients with microscopic colitis (25). Because of the limitations of microscopic examination of stool for leukocytes outlined in the Introduction, this test was not performed in our patients' stool specimens collected quantitatively over 2–3 days. Thus, although this discrepancy cannot be resolved at this time, it is possible that the fecal leukocytes reported to be present in the previous studies of microscopic colitis (25, 26) in fact were mononuclear cells or eosinophils, rather than neutrophils. It is unlikely that neutrophils can be seen by fecal microscopy but not detected by the lactoferrin test, inasmuch as the Leuko-Test has routinely been more sensitive than microscopy in studies of acute diarrhea, and the threshold number of neutrophils per high power microscopy field that incites a positive Leuko-Test is one (3).

The utility of a positive fecal test for lactoferrin in pa-

tients with acute diarrhea has come to light recently as a possible means of reducing the cost and improving the yield of subsequent diagnostic tests, mainly bacterial cultures (9, 12). Based on the excellent sensitivity, specificity, and predictive value of the Leuko-Test for neutrophilic intestinal (mainly colonic) inflammation found in this study, the same may be true of chronic diarrhea. A positive fecal lactoferrin test discovered at the outset of investigation of a patient with chronic diarrhea would appropriately direct the workup to an endoscopic procedure of the colon to inspect and randomly biopsy the mucosa. If this was unrevealing, a small bowel barium X-ray looking for Crohn's disease or perhaps celiac sprue probably would be the most useful next step. The Leuko-Test also may be beneficial in confirming a diagnosis of inflammatory bowel disease (e.g., when suspected by X-ray) or determining whether such disease is active (25). It is also worth reiterating that in our series of 103 of patients with chronic diarrhea, a negative fecal lactoferrin test had a 99% predictive value for ruling out inflammatory bowel disease.

There is only one other study of which we are aware that has investigated the significance of fecal leukocytes in a cohort of patients with chronic diarrhea (28). In this Brazilian study, 42 of 1680 patients with "chronic bowel diseases" (2.5%) had fecal leukocytes detected in stool by microscopy. Thirty-five had chronic idiopathic inflammatory bowel disease (ulcerative colitis in 33, Crohn's disease in 2), four had colonic carcinoma, two had amebic colitis, and one had eosinophilic colitis. Because the final diagnoses in the patients without fecal leukocytes were not investigated in this study, the sensitivity and negative predictive value of fecal leukocyte microscopy in the setting of chronic diarrhea remains unknown. In our study, 10 of 103 patients (10%) were diagnosed with ulcerative colitis or Crohn's disease, nine of which were identified on initial testing using the fecal lactoferrin test. In contrast, only 35 of 1680 pa-

tients in Brazil (2%) had inflammatory bowel disease detected by the presence of fecal leukocytes. Acknowledging the possibility that more patients in Brazil may have non-inflammatory infectious chronic diarrhea relative to our population, and that our institution is a U.S. tertiary care hospital, nevertheless, the lower yield of chronic inflammatory diagnoses by microscopy versus that discovered by the Leuko-Test in part may relate to a lower sensitivity for the detection of fecal neutrophils by microscopy.

In our study, stools from the individuals in the positive and negative control groups and from patients found to have inflammatory bowel disease were tested in duplicate. Only one paired test gave contradictory results. This was from a patient with active ulcerative colitis who initially was found to have a negative fecal lactoferrin test but who had a positive result twice when the same fecal specimen (stored frozen and thawed) was retested on two separate occasions. Although the initial test result represents a false negative relative to the patient's diagnosis (due either to failure of the test or the technician), it is possible that from a technical standpoint, it was a true negative, and that the difference between the negative and positive results reflects variation in the concentration of lactoferrin in different areas of the same sample. The stool specimen from this patient had a soft rather than liquid consistency, so that lactoferrin may not have distributed evenly despite our best attempts at homogenization. Confirming that this patient had lactoferrin in stool upon repeat testing is important because it suggests that all patients with chronic diarrhea caused by active inflammatory bowel disease do have neutrophilic cell products in stool that can be detected immunologically, but that in rare cases duplicate testing may be necessary. However, with the excellent diagnostic accuracy of the initial Leuko-Test results found in this study (Table 2), it seems that the benefits of performing the test in duplicate (*i.e.*, from two different areas of the stool specimen, analogous to the method of Hemoccult testing) in all patients would not outweigh the disadvantage of the added cost of test materials (albeit, this is only \$5 per test). Alternatively, such duplicate testing may be indicated for an initial negative result if the clinical suspicion for inflammatory bowel disease is high, and the consistency of the diarrheal stool specimen is soft rather than liquid.

Although the hemoccult test was developed to specifically screen for colon cancer, it is often used more generally to detect fecal occult blood from any source, the way guaiac based methods have been used for >130 yr (29). In the setting of acute diarrhea, the presence of gross blood in stool usually is equated with the presence of intestinal inflammation (mainly colitis). Except for malignancy, the same probably is true for diseases causing chronic diarrhea. However, because not all patients with inflammatory bowel disease have gross bleeding, and as shown in this study, only 64% even have occult bleeding, Hemoccult testing *per se* will have a low sensitivity for detecting Crohn's disease or ulcerative colitis in patients with chronic diarrhea. More-

over, inasmuch as many chronic diarrhea patients without intestinal inflammation also have positive fecal Hemoccult tests (24% in this study), the specificity is also poor.

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Established and Emerging Biological Activity Markers of Inflammatory Bowel Disease

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ABSTRACT

Assessment of disease activity in inflammatory bowel disease (IBD), *i.e.*, ulcerative colitis (UC) and Crohn's disease (CD), is done using clinical parameters and various biological disease markers. Ideally, a disease marker must: be able to identify individuals at risk of a given disorder, be disease specific, mirror the disease activity and, finally, be easily applicable for routine clinical purposes. However, no such disease markers have yet been identified for IBD. In this article, classical disease markers including erythrocyte sedimentation rate, acute phase proteins (especially orosomucoid and CRP), leukocyte and platelet counts, albumin, neopterin, and β_2 -microglobulin will be reviewed together with emerging disease markers such as antibodies of the ANCA/ASCA type, cytokines (*e.g.*, IL-1, IL-2R α , IL-6, IL-8, TNF- α , and TNF- α receptors) and with various adhesion molecules. It is concluded that none of the pertinent laboratory surrogate markers of disease activity in IBD are specific or sensitive enough to replace basic clinical observation such as the number of daily bowel movements, general well-being, and other parameters in parallel. Further studies are highly warranted to identify and assess the clinical importance and applicability of new laboratory markers for the diagnosis or the disease activity of IBD. (*Am J Gastroenterol* 2000;95:359–367. © 2000 by Am. Coll. of Gastroenterology)

INTRODUCTION

Chronic inflammatory disorders of the intestine, *i.e.*, inflammatory bowel disease (IBD), comprise two major disease entities: ulcerative colitis (UC) and Crohn's disease (CD).

Numerous clinical activity indices are used in relation to IBD (1), but they all give only indirect assessments of disease activity, and none is accurate in assessing inflammatory activity as found by histopathological or endoscopic examination. In UC the most widely used clinical parameters are the number of daily bowel movements and the presence of bloody stools, whereas albumin, hemoglobin, erythrocyte sedimentation rate (ESR), and elevated acute-phase protein levels are commonly used biological param-

eters. For CD disease activity, daily bowel movements and the presence of abdominal pain and/or discomfort are the parameters most frequently used clinically, whereas serum levels of orosomucoid and C-reactive protein (CRP) are classical laboratory tests (2). Definition of disease activity is a major problem in IBD. Optimally, a disease activity index should reflect both clinical measures such as fever, diarrhea, abdominal pain, gut inflammation, general well-being, and quality-of-life. Above all, it should reflect the patient's overall condition. Several compound indices have been used: *e.g.*, Crohn's Disease Activity Index (CDAI), True-love, and Harvey-Bradshaw, but they are almost invariably too complex to be applied in everyday clinical practice. To some extent the degree of inflammation correlates with the well-being of the patient (3) and may, for research purposes, be used as a "disease activity" measurement. However, whether the inflammation is best determined from systemic factors or from the bowel itself is controversial. Furthermore, the many indices indicate that none of them to date is adequately reliable and valid. Hence, evaluation of the potential use of laboratory markers must, until a "gold standard" for disease activity is found, rely on an overall assessment of the severity of disease. References to disease activity in this review are made to the various activity indices or parameters used in the study in question.

Various biological markers of inflammation have been used for the diagnosis and assessment of prognosis and success of therapy in IBD. Quantitative assessment of the degree of active inflammation is of great clinical importance, and the requirements of biological disease markers are therefore multiple. A disease marker must ideally be able to identify individuals at risk for a given disease, be disease-specific, *i.e.*, be able to distinguish IBD patients from other patients, distinguish UC from CD, and be able to monitor disease activity. It should also be capable of monitoring the effect of treatment and should give prognostic information. Furthermore, markers should be easily applicable for screening purposes. The demands imply both simple and minimally invasive sampling as well as fast and simple analysis procedures. Finally, disease markers should be inter- and intraindividually reproducible with regard to

both laboratories and patients. No marker launched so far encompasses all of these characteristics. Even when analyzing the results of a broad panel of surrogate markers with computer software, these aims cannot be met at the present time. The commonly used laboratory markers are, even at their best, not specific with respect to inflammation in the gut, as they reflect the degree of inflammation in the whole body. They need to be carefully evaluated together with the patient history and clinical examination as well as with information from diagnostic imaging and endoscopy (1).

To identify appropriate surrogate markers of disease or disease activity, several attempts have been made to screen and to investigate IBD patients during the natural course of the disease (individuals at risk, as well as subjects undergoing therapy) (4, 5). Both "classical" and "emerging" biological activity markers of relevance for IBD will be reviewed in the succeeding part of this article.

"CLASSICAL" MARKERS

Erythrocyte Sedimentation Rate (ESR) and Acute Phase Proteins

ESR, which is the rate at which erythrocytes fall through plasma, is a classical marker of the acute phase response, and is still widely used (6). It varies with plasma protein concentrations and the hematocrit value, and in IBD provides a crude and rapid assessment of the plasma protein alterations of the acute phase response. Thus, in general, the ESR is an indirect measurement of plasma acute phase protein concentrations, and is greatly influenced by the size, shape, and number of erythrocytes as well as by other plasma constituents such as immunoglobulins. As the levels of many serum proteins vary in IBD, and as some have long half-lives, the ESR is not necessarily accurate and timely in reflecting major changes in the clinical state. The ESR may take several days to decrease even when rapid clinical improvement occurs. In UC where clinical, endoscopic, and histological grading of disease severity may be used to assess the overall disease activity, the correlation between ESR and disease activity is good (7, 8). ESR is, however, not useful in distal proctitis, presumably because of the relatively small intestinal area involved in the disease process in this case. In CD the use of ESR also appears to be questionable. ESR does actually increase with increasing disease activity (9), but it correlates to a higher degree with colonic CD involvement than with CD localized to the small bowel (10).

Classical acute phase proteins are orosomucoid (α_1 -acid glycoprotein), CRP, fibrinogen, lactoferrin, serum amyloid A, and α_1 -antitrypsin. The levels of circulating orosomucoid and CRP both correlate with disease activity of IBD as assessed by standard indices (2, 9, 11). Furthermore, circulating orosomucoid levels correlate with the protein loss into the gut (11), but the 5-day half-life in serum limits its usefulness as an indicator of improvement in disease activity. CRP has a significantly shorter half-life of 19 h, which

explains the more rapid decrease in serum concentration after reduction of inflammatory activity in IBD (12). Preliminary results indicate α_1 -antitrypsin and lactoferrin in feces as both sensitive and bowel-specific markers of disease activity of IBD (13).

Leukocytosis

Active IBD often causes an elevation of the white blood cell (WBC) count, predominantly due to an increase in polymorphonuclear leukocytes (PMNs) (14). Although systemic glucocorticoid treatment may specifically increase the granulocyte count, other immunosuppressants may lower it. In CD, the presence of abscesses may lead to elevated PMN counts, while complicating lymphangiectasia results in lymphocyte loss through the intestinal wall, and thus a reduced number of circulating T cells (15). In some UC patients a distinct leukocytosis occurs, which, at the tissue level, may be associated with the healing phase (16). However, in general, this marker is not useful for assessment of IBD activity in clinical practice.

Platelets

In IBD, the platelet count correlates with disease activity. Platelet levels exceeding $400 \times 10^9/L$ are more common in severe than in mild or moderate UC, and in CD the platelet count correlates with CDAI (17, 18). As the platelet count has a fairly wide normal range and a small range of abnormality, and as other compounding factors such as hemorrhage of any sort can raise the platelet count, this parameter is not widely used in clinical practice.

Albumin

Serum albumin levels decline in active disease, although other factors such as protein loss from the gut, as well as malnutrition, may influence albumin concentrations (19). Extremely low serum albumin levels can be used as a predictor of the outcome of medical treatment in severe exacerbations of UC (*i.e.*, patients eventually requiring surgery) (20).

Neopterin

Neopterin, a pteridine intermediate metabolite in the synthetic pathway of biopterin, is synthesized and released from monocytes/macrophages upon nonspecific stimulation. Its value as a marker of T-cell, monocyte, and macrophage activation has been shown in a number of disorders including various infectious, inflammatory, autoimmune, and malignant diseases (21). Along with the known activated cellular immunity in IBD, both elevated urine and serum levels of neopterin are seen. The level of neopterin in urine as well as in serum has been shown to correlate with disease activity of both CD (22, 23) and UC (24). Increases of neopterin levels, however, are not IBD-specific, as it simply reflects a condition in which T cells, monocytes, or macrophages are activated. Measurements of this marker in urine or serum may, however, provide additional information about the grade of activated cellular immunity in IBD.

β_2 -Microglobulin

β_2 -Microglobulin is a low molecular weight protein constituting the light chain of HLA class I antigen on the cell membranes of all nucleated cells, especially abundant in lymphocytes, macrophages, and endothelial cells. Elevated levels of this marker are thought to reflect increased release of the protein primarily from activated T-cells and neutrophils (25). β_2 -Microglobulin is passively filtered through renal glomeruli with almost all being reabsorbed and metabolized in the proximal tubuli of the kidneys (26). Measurements of serum β_2 -microglobulin have primarily been used as a sensitive indicator of renal function. Elevated levels are found in patients undergoing chronic hemodialysis and in various disorders including autoimmune disorders (26), malignant disease (primarily hematological) (27), liver disease (28), sarcoidosis (29), cardiomyopathy (30), and coeliac disease (31).

The use of β_2 -microglobulin as a disease activity marker in IBD has been only sparsely investigated. To our knowledge, only one study involving seven UC patients has been published, and no correlation between β_2 -microglobulin levels and clinical disease activity was found (32). In CD there are also very few studies involving relatively small numbers of patients, and the results are somewhat conflicting. In one study, elevated levels of β_2 -microglobulin were found in CD correlating with clinical disease activity and distinguishing active disease from quiescent disease (33). These findings were confirmed in two later studies (34, 35). Yet another study did not find any correlation to clinical disease activity, no difference between active and quiescent disease, and no difference between patients and controls (36). Also, no correlation between β_2 -microglobulin levels and clinical disease activity was found in a small study involving only children with CD and UC (32).

The known involvement of cellular immunity in CD could indicate the possibility that β_2 -microglobulin is applicable as a marker of disease activity. However, further studies are needed until any conclusion can be drawn upon this question.

"EMERGING" MARKERS

ANCA/ASCA

Antineutrophil cytoplasmic IgG antibodies (ANCA) are classically associated with vasculitis, especially Wegener's granulomatosis, in which measurements of serum levels of ANCA are used for diagnostic, monitoring, and prognostic purposes. In addition, ANCA are found in other chronic inflammatory disorders, most notably in rheumatoid arthritis and in UC (37, 38). In both conditions, the immunofluorescence pattern is of the perinuclear ANCA (pANCA) staining type. The exact identity of the reactive antigen(s) has remained elusive, but the autoantigens are clearly distinct from the specificities commonly involved in vasculitis (proteinase-3 and myeloperoxidase) (39, 40). A characteristic feature of the pANCA found in UC appears to be the

disappearance of reactivity on DNase-treated neutrophils (41, 42), but the methodology is not yet standardized. Recently, the pANCA antigens have been tentatively identified as the non-histone chromosomal proteins, HMG1 and HMG2 (43). No apparent correlation between the presence and titer of pANCA and IBD duration, activity, localization, patient age, sex, or treatment has been demonstrated (40). However, pANCA is much more common in UC (50–70%) than in CD, in which the prevalence (5–10%) is only slightly higher than that found in healthy individuals (3–4%) (44). This 5–10% might designate a clinically distinct subpopulation of CD patients (45).

Although the presence of ANCA is characteristic of UC, another class of antibodies (IgG and IgA) directed against the phosphopeptidomannan part of the cell wall of *Saccharomyces cerevisiae* (baker's yeast) (anti-*Saccharomyces cerevisiae* antibodies, ASCA) are emerging as serological markers for CD (46). Various studies have addressed the fine specificity of these antibodies, and differences in reactivity with extracts from different yeast strain mannans have been demonstrated (46, 47). Despite the lack of standardization within this area, in some studies elevated titers of IgG and/or IgA ASCA have been found in 50–80% of CD patients but rarely (<10%) at similar titer levels in UC patients or in healthy controls (<5%) (42, 43, 47). ASCA might therefore be a serological marker of high diagnostic specificity for CD, although its diagnostic sensitivity is intermediate. In contrast to pANCA, the ASCA titer may reflect disease activity, as it decreases after resection of damaged gut tissue (42), but further studies are needed to confirm and to elaborate upon this. Family studies indicate that ASCA may be used to identify individuals at risk for CD, as ASCA is present with increased frequency as compared to that in individuals with no family history of CD in first degree relatives of IBD patients (48).

Sensitivities are too low to advocate the ANCA or the ASCA tests as the only diagnostic tool in UC or CD (49–51), but the combined measurement of pANCA and ASCA may be used advantageously in the subclassification of IBD patients with indeterminate colitis (44, 47). Both antibody specificities are measured by traditional quantitative solid phase immunosorbent assays, and they are highly specific (>90%) for both UC and CD with a disease sensitivity around 50% in both cases. The differential diagnostic specificity of ANCA for UC and ASCA for CD is unique among the many potential markers covered in the present review. Future prospective studies using standardized assays and prolonged follow-up periods are needed to extend the indications for diagnostic applications of these antibodies.

Cytokines

The inflamed intestine in IBD is characterized by T cell activation and mucosal influx of inflammatory cells, partly mediated by an increased local release of cytokines and chemokines and partly as the relative increased expression of specific chemokine receptors. Proinflammatory cytokines

produced by activated phagocytes, especially macrophages and T lymphocytes, are of central importance in the chronic inflammation of IBD. As related receptors with different affinities and cross-reactive binding capacities are present on leukocytes, relative differences in the receptor distribution and receptor affinity for specific chemokines may significantly influence which cells ultimately are attracted and activated in IBD.

The expression of proinflammatory cytokines in the intestinal mucosa from IBD patients is markedly enhanced, although not always accompanied by increased concentrations of cytokines in the circulation. Future investigations will clarify the significance of impairments of the cytokine network for the initiation and progression of IBD. The complex pattern of chemokine-orchestrated chemotaxis, leukocyte activation, granulocyte exocytosis, increased production of metalloenzymes, and up-regulation of respiratory burst activity indicate that chemokines increase and perpetuate inflammation and chronic intestinal tissue destruction in IBD by a variety of different mechanisms.

TNF- α AND RECEPTORS. TNF- α is secreted primarily by activated macrophages and monocytes (52). Treatment with anti-TNF antibodies in both CD and UC refractory to conventional medical treatment has shown promising results (53–55), indicating that this cytokine has a pivotal role in IBD.

Increased numbers of TNF- α secreting cells have been found in inflamed mucosa in IBD (especially CD) (56). Spontaneous TNF- α production from isolated lamina propria mononuclear cells from both inflamed IBD mucosa and macroscopically uninvolved CD mucosa has been found to be increased when compared to that in normal mucosa (57, 58). In one study, a close correlation of TNF- α production with the degree of tissue involvement and mucosal inflammation was found (57). Increased peripheral blood mononuclear cell production of TNF- α has also been revealed in both CD and UC (59).

Elevated serum TNF- α (highest in colonic CD) correlating with clinical and laboratory indices of disease activity, and in UC associated with severe disease leading to surgery, has been described (59–62). One study, however, did not find any differences in serum TNF- α levels between IBD and functional bowel disease (63), and another study found only a statistically insignificant tendency toward higher TNF- α levels in active IBD as compared with that in healthy volunteers (64).

The results are also somewhat conflicting regarding measurement of TNF- α in stools from IBD patients. Significantly increased stool concentrations in both active CD and UC have been found (65, 66) with levels decreasing to those of healthy controls in inactive disease (67). One report showed increased TNF- α concentrations in stools from active UC, whereas the levels in both CD and inactive UC were similar to healthy and inflammatory controls. Furthermore, a significant reduction in TNF- α concentrations after

clinical resolution in both UC and CD has been described (67).

Circulating levels of the soluble receptors TNF-R I (p55) and TNF-R II (p75) have been described as being significantly increased, correlating with disease activity in both CD and UC in two studies (62, 68). Another study could not reproduce this finding in active CD (69). Significantly higher levels of both soluble TNF-R I (p55) and TNF-R II (p75) in urine from patients with active UC and CD, both in comparison to inactive patients and healthy controls, have been described, and the levels correlated with clinical disease activity (70).

From the available literature TNF- α does not seem to be promising as a marker of activity in IBD (64), and the large interindividual variation of the levels remains problematic. Stool measurements of TNF- α may be more promising, but it remains to be confirmed whether stool levels in UC, CD, or both are valid in the monitoring of disease activity.

The use of soluble TNF receptors (both serum/plasma and urine) as activity markers in IBD might be reliable, but further studies must be performed before general implementation can be advocated. The noninvasive measurements, especially, could be interesting as a tool for the monitoring of disease activity (71).

IL-1 AND IL-1RA. Interleukin-1 (IL-1), a proinflammatory cytokine, and its natural counter receptor, IL-1 receptor antagonist (IL-1ra), play major roles in the regulation of inflammation (72).

IL-1ra inhibits the proinflammatory actions of IL-1 by binding to its receptors on target cells. IL-1ra has no known agonist effects. Two IL-1ra variants have been described: a secreted form produced by mononuclear cells, and an intracellular form found in keratinocytes and other epithelial cells. The findings of high plasma and tissue levels of IL-1ra in patients with various inflammatory diseases such as rheumatoid arthritis and IBD indicate that IL-1ra may be part of the host's homeostatic mechanisms aimed at down-regulating inflammation. Several studies have shown that the IL-1ra/IL-1 ratio decreases with increasing IBD activity (73). Thus, it is suggested that a deficit in endogenously produced IL-1ra is of pathogenic importance in IBD. Furthermore, one study has described increased levels of IL-1ra in active CD and UC that were, however, not as pronounced as the increase of IL-1 compared with inactive IBD and with infectious colitis patients (74).

IL-2R. T cell activation and expansion of T cell clones involves IL-2 generation by T cells themselves, and the activated T cells further shed IL-2-receptor (IL-2R) in a soluble form into the circulation. In IBD, the measurement of elevated soluble IL-2 receptor levels has potential advantages over acute phase proteins in that it more accurately reflects the underlying immunopathogenic process (75). The IL-2R is more stable and therefore possibly more reliable for detection in the circulation than IL-2 and consists of three

chains: p55 (CD25, α chain); p75 (CD122, β chain); and p64 (CD132, γ chain). It has been shown that levels of IL-2R α correlate positively with increasing disease activity in both UC and CD (76). Although IL-2R β levels also increase with increasing disease activity in CD, circulating IL-2R β decreases with increasing disease activity in UC, which indicates different mechanisms in UC and CD (77). Increased IL-2R γ levels are found in active CD but not in UC (78). Finally, a study of the IL-2R (α -chain) in colonic lamina propria have shown elevated concentrations in UC but not in CD mucosa as compared to control colonic mucosa (79).

IL-6. IL-6, which is produced by macrophages and T cells (80), promotes the differentiation of B cells, acts as cofactor in the stimulation of T cells, and induces hepatic release of acute phase proteins. Several studies have shown elevated serum levels of IL-6 in active CD but not in UC, in which levels not always differed from those of healthy controls (81, 82). Immunohistochemical studies on its expression in intestinal tissue reveal only infrequent colonic epithelial expression, irrespective of the presence of inflammation (83). However, increased levels of IL-6 mRNA are found in actively inflamed IBD mucosa as compared to healthy controls.

Elevated circulating levels of IL-6 receptor (IL-6R) have been detected both in active UC and in CD when compared to inactive disease stages. It has been proposed that soluble IL-6R affects the bioavailability of its ligand during inflammation (84, 85). In contrast to other soluble cytokine receptors that block the cellular actions of their physiological cytokine ligands, the opposite is the case for IL-6. Thus, a genetically engineered, soluble IL-6R augments the IL-6 induction of acute phase proteins such as α_1 -antichymotrypsin and haptoglobin in human hepatoma cells *in vitro*, at both the mRNA and protein levels (86). This indicates that sIL-6R acts as an agonist of the IL-6 mediated cell responses. It is unknown whether sIL-6R enhances all IL-6 functions, but the positive correlations between serum levels of CRP and sIL-6R, as well as IL-6 itself, support this hypothesis (85).

IL-8. IL-8, which is produced by activated PMNs, macrophages, and epithelial cells (which are all abundant in inflamed IBD intestine), is an important inflammatory mediator for phagocyte chemotaxis and especially that of PMNs (87). Chemotaxis is essential for initiation and perpetuation of the inflammatory cascade, as PMN accumulation and activation may cause granulocyte exocytosis, increased production of metalloenzymes, and up-regulation of the respiratory burst (14). Thus, the recruitment of PMNs from the circulation and to the inflamed intestinal lesions of IBD occurs along a chemotactic gradient, and a recent study has stressed the importance of IL-8 in this respect (88). It has been reported that serum IL-8 is a poor marker of disease activity in IBD (89). In one study, increased circulating

levels of IL-8 were found in active UC only, and not in CD (90). In inflamed intestinal segments of IBD, however, elevated levels of IL-8 have been detected (91–93). Furthermore, the intestinal gene expression of IL-8 has been found to be elevated even in inactive IBD (91, 94), with high levels of IL-8 mRNA in inflamed segments, especially in UC (95). A recent study of IL-8 in feces and rectal dialysate fluid has shown elevated values in active UC as compared to those of controls, whereas patients with CD were not investigated (96).

New treatment principles of IBD with IL-10 or neutralizing monoclonal antibodies against TNF may inhibit IL-8 production and reduce the intestinal cytokine mRNA level in IBD (97, 98). However, measurement of IL-8 in feces and rectal dialysate might be a possibility for assessment of IBD activity, whereas measurement of IL-8 in biopsy specimens seems to be too inconvenient for clinical purposes.

Cell Adhesion Molecules

Cell adhesion molecules (CAMs), expressed by immune cells, endothelial cells, and epithelial cells are exerting their effects locally in the bowel mucosa (99). Some CAMs are also found as soluble molecules in the blood, but it is not known whether these circulating CAMs take active part in the inflammatory response, or whether they are inactive byproducts in which local activation causes endothelial cells and leukocytes to shed membrane-bound CAMs (100–106). It has been suggested that the soluble forms act in an anti-inflammatory manner by binding to their receptors on circulating leukocytes. Circulating CAMs might, in any case, reflect the degree of inflammation in the intestines and theoretically, therefore, may be used as activity markers of inflammation in IBD.

The circulating CAMs investigated so far include soluble intercellular adhesion molecule-1 (sICAM-1), vascular cell adhesion molecule-1 (sVCAM-1), sE-selectin, and sP-selectin. These four CAMs are all expressed on luminal parts of endothelial cell membranes and are thus localized in the blood vessel lumen. Another studied soluble cell adhesion molecule is L-selectin, which is expressed on leukocytes and shed upon their activation (107).

Compared to that in healthy controls, the sICAM-1 concentration is increased in IBD, both in serum (100–102) and in plasma (103). Significantly higher levels of sICAM-1 in active UC (101, 103) and active CD (101–103), as compared to inactive IBD, have been revealed, and increased plasma levels were found in colectomized UC patients with pouchitis as well (103). Serum concentrations of sICAM-1 correlated with levels of orosomucoid and C-reactive protein (CRP) (100–102), but did not increase the precision of activity assessment compared with the clinical and endoscopic indices routinely applied. In one study, serum sICAM-1 correlated directly with von Willebrand factor levels and inversely with albumin and hemoglobin (the latter only in active UC) (102). This study also showed a correlation between serum levels of sICAM-1 and the

amount of ICAM-1 in colonic tissue assessed semiquantitatively by immunohistochemical staining procedures. ICAM-1 is up-regulated *in situ* in inflamed mucosa in IBD.

sVCAM-1 is found in higher circulating concentrations in serum in IBD patients as compared to controls (100, 102), although with a weak correlation with disease activity (102). Like sICAM-1, sVCAM-1 correlates directly with the level of von Willebrand factor, platelet count, and ESR, and correlates inversely with hemoglobin and albumin (102). Studies on plasma did not show these differences.

In contrast to the studies of sICAM-1 and sVCAM-1, which almost uniformly find increased serum concentrations in active IBD, the selectin measurements are somewhat contradictory. To date, only one article deals with serum sP-selectin, which was found to be increased in IBD when compared to that in healthy controls (100). sE-selectin levels in serum or plasma are found, in some reports, to be increased in IBD (102, 103), whereas others do not report any differences among patients with UC, CD, and controls (100, 104). In plasma samples, increased sE-selectin levels were found in active UC, active CD, and in colectomized UC patients with pouchitis (103). sE-selectin also correlated with CRP in this study. No other study has investigated the correlation of E-selectin with disease activity or acute phase proteins. Circulating L-selectin (sL-selectin) levels are rising with increased disease activity in UC, whereas no such correlation is found in CD (107). There is no pathophysiological explanation for the disease-specific regulation of sL-selectin, and so far no difference in the expression of endothelial cell L-selectin ligands has been found (84). Soluble L-selectin is increased in various other inflammatory disorders such as sepsis and rheumatoid arthritis (105, 108), and possibly reflects overall leukocyte activation. Interestingly, UC patients with quiescent disease stages have significantly lower sL-selectin levels than controls (107).

It is unknown whether the ligands of ICAM-1, *i.e.*, the β_2 integrins, are present as unbound proteins in the blood. However, studies of their expression on circulating leukocytes have been conducted using fluorescence-activated cell sorting (FACS) methods. Although one study did not find differences in the number of β_2 integrin molecules on leukocytes or the ratio of β_2 integrin-positive leukocytes among UC, CD, and controls (106), another showed that remission in UC (due to medical treatment) was accompanied by a decrease in the ratio of T cells positive for the β_2 integrin lymphocyte function-associated antigen-1 (LFA-1) (109). Preliminary results indicate that the number of lymphocytes expressing the β_2 integrin CD11c/CD18 (p150.95) is increased in UC when compared to CD and nondiseased controls, and that CD18 is not present on PMNs in UC in contrast to PMNs from healthy individuals (B. Vainer, unpublished results).

For all the CAMs studied as either soluble or leukocyte-bound proteins, significant overlaps between UC and CD, between IBD and controls, and between inactive and active IBD are found (99–107). This fact renders CAMs poor

markers of IBD or IBD activity in the blood (serum, plasma, or isolated leukocytes). Importantly, measuring soluble CAMs in serum or plasma with the currently available methods is neither easier to perform nor more convenient to the patients, and does not appear to add new information to the paraclinical or clinical parameters already in use.

CONCLUSIONS

Knowledge of the severity and extent of inflammation in IBD provides a means of choosing a rational therapeutic strategy. During the past three decades, several clinical, laboratory, and combined indices have been proposed for the assessment of disease activity in IBD; and refinements in radiological methods and the availability of endoscopy and biopsy have facilitated the accurate measurement of the extent and severity of IBD. In relapsing conditions, however, the use of such procedures is limited by the radiation exposure and/or by the relatively invasive nature of the techniques. The assessment of disease activity among IBD patients by the use of various biological indices may benefit from new approaches. The most up-to-date approach to monitoring can be summarized as follows: The classical acute phase proteins, orosomucoid, and CRP are easily applied in the clinical routine situation, together with emerging activity markers such as circulating IL-1, IL-2R α , IL-6 and its receptor, neopterin, and TNF- α receptors in urine, possibly TNF- α and IL-8 in stools, and IL-8 in rectal dialysate fluid. ANCA and ASCA may lead to more accurate differential diagnosis of UC and CD. Finally, it should be emphasized that further studies are highly warranted to assess the clinical importance and applicability of these new markers for reassurance of diagnosis or disease activity.

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Serologic Markers in Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is an enduring disease involving mostly young people, with symptoms of bloody diarrhea and abdominal cramps. Several antibodies have been associated with IBD, the 2 most comprehensively studied being autoantibodies to neutrophils (atypical perinuclear anti-neutrophil cytoplasmic antibodies) and anti-*Saccharomyces cerevisiae* antibodies. This review focuses on the value of these antibodies for diagnosing IBD, differentiating Crohn disease from ulcerative colitis, indeterminate colitis, monitoring disease, defining clinical phenotypes, predicting response to therapy, and as subclinical markers. Pancreatic antibodies and newly identified anti-microbial antibodies (anti-outer membrane porin C, anti-I2, and anti-flagellin) are also reviewed.

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Inflammatory bowel disease (IBD)¹ embodies a spectrum of disorders that affect the gastrointestinal tract, the 2 major entities being Crohn disease (CD) and ulcerative colitis (UC). IBD is a lifelong disease involving mostly young people, often in a severe way. Although the etiology of IBD is unknown at present, it is believed to be an immunologically mediated disease in a genetically susceptible host. The picture that emerges is that IBD results from an aberrant immune response and loss of tolerance to the normal intestinal flora, leading to chronic inflammation of the gut. This idea is supported by the occurrence of antibodies directed to microbial antigens (see below) and by the identification of *CARD15* as a gene conferring susceptibility to CD (1). *CARD15* plays a role in the recognition of bacterial structures and in the subsequent defense against these bacteria through nuclear factor- κ B.

Several (auto)antibodies have been described in IBD. The 2 most intensively studied antibodies are autoantibodies to neutrophils [atypical perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCAs)] and anti-*Saccha-*

romyces cerevisiae antibodies (ASCAs). This review summarizes the current knowledge about the putative clinical value of these 2 antibodies in IBD. Pancreatic antibodies and newly identified anti-microbial antibodies (anti-outer membrane porin C, anti-I2, and anti-flagellin) are also reviewed.

Clinical Presentation and Diagnosis of IBD

CD patients may present with almost any gastroenterologic symptom depending on the site of the disease. Colonic disease (either alone or together with small intestine) is more common than disease in the terminal regions of the ileum or cecum and usually presents with chronic abdominal pain and diarrhea. In more severe forms, a mass may be present in the right iliac fossa. Patients with CD can have typical perianal lesions, such as ulcers or multiple fistulas. In CD, serum concentrations of C-reactive protein (CRP) correlate well with disease activity and with other markers of inflammation as the CD activity index (2). Increased CRP (>45 mg/L) in patients with IBD predicts with a high certainty the need for colectomy (2). Patients with UC suffer from bloody diarrhea or rectal bleeding and tenesmus because of the rectal involvement.

There are several structural differences between CD and UC (3). In UC, the disease is restricted to the rectum and colon. The lesions are continuous and restricted to the mucosa. There is muscular thickening, mucin depletion, and glandular damage. In CD, the disease may affect any part of the gastrointestinal tract. The lesions are discontinuous and transmural (fissure, abscess, fistula). There may be fibrosis (stenosis) and lymphoid ulcers. Histiocytic granulomas are the hallmark of CD, but are found in only ~60% of cases.

Diagnosis of IBD and differentiation between CD and UC can be made accurately in most patients based on the patient's history and physical examination, ileocolonoscopy examination, biopsy, double-contrast barium enema examination, and microbiology. The differential diagnosis includes irritable bowel disease; infective

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¹ Nonstandard abbreviations: IBD, inflammatory bowel disease; CD, Crohn disease; UC, ulcerative colitis; P-ANCA, perinuclear anti-neutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody; CRP, C-reactive protein; BPI, bactericidal permeability increasing; and HMG, high-mobility group.

diarrheas attributable to *Salmonella*, *Campylobacter*, or *Shigella*; lymphoma; carcinoma of the colorectum; and inflammation caused by ischemia or irradiation (3, 4).

Not all endoscopic biopsy specimens can be firmly assigned to either CD or UC. This is particularly the case for colonic biopsies from cases of proctitis. In ~10% of patients with IBD, the disease cannot be classified as CD or UC and the final diagnosis is "indeterminate colitis", even if the whole colon is removed (5). The diagnosis of indeterminate colitis is usually a temporary diagnosis, and many patients with indeterminate colitis will be diagnosed with either UC or CD over time.

Disease behavior in CD can be classified into primary fibrostenotic (rather indolent behavior), primary perforating (abscesses and fistulas; aggressive behavior), and primary inflammatory (5). UC can be classified into pancolitis (associated with greater severity and greater risk of malignant change), left-sided colitis (no local therapy possible), distal colitis, and proctitis (may be resistant to local therapy) (5).

Autoantibodies to Neutrophils: ANCAs and Atypical P-ANCAs

ANCAs were originally associated with primary small vessel diseases, such as Wegener granulomatosis, microscopic polyangiitis and its renal limited variant (pauci-immune crescentic glomerulonephritis), and Churg-Strauss syndrome (6). ANCAs are classically screened by indirect immunofluorescence using ethanol-fixed neutrophils, as agreed on in an international consensus statement (7). Indirect immunofluorescence shows 2 major staining patterns: a cytoplasmic granular (C-ANCAs) and

a perinuclear pattern (P-ANCAs). The C-ANCA pattern shows granular cytoplasmic fluorescence, frequently accentuated between the nuclear lobes. C-ANCAs are primarily present in sera from patients with Wegener granulomatosis and mainly recognize proteinase-3. The P-ANCA pattern shows a fine homogeneous rim-like staining of the perinuclear cytoplasm (just around the nucleus). Nuclear extension can be present. P-ANCAs are present in patients with microscopic polyangiitis and recognize myeloperoxidase. However, P-ANCA staining is also seen with antibodies to other neutrophil enzymes and with anti-nuclear antibodies (a neutrophil-specific anti-nuclear antibody or wrong reading because of the presence of anti-nuclear antibodies).

ANCAs have also been reported in patients with chronic inflammatory disorders, such as UC (60%–80%) (8, 9), primary sclerosing cholangitis (88%) (10), autoimmune hepatitis (81%) (11), and to a lesser extent, CD (5%–25%). In these disorders, a (atypical) P-ANCA staining pattern is usually found. The antigen is not myeloperoxidase. The atypical P-ANCA is characterized by a broad inhomogeneous rim-like staining of the nuclear periphery (12).

Autoantigens Recognized by Autoantibodies to Neutrophils (Atypical P-ANCAs) in IBD

Overviews of the various studies (13–28) that have attempted to identify the antigenic species recognized by atypical P-ANCAs in UC and CD patients and in controls are given in Tables 1 through 3. The antigens that have been studied are located in the granules, the cytosol, and/or the nuclei or nuclear periphery. The serine pro-

Table 1. Prevalence of autoantibodies to specific antigens in UC patients.

Study	Method	Healthy controls		Patients, n	Positive antibody test, %										
		n	SD ^a		ANCA ^s	BPI	β -Glu ^b	Cathepsin G	Elastase	Lysozyme	Lactoferrin	Catalase	α -Enolase	HMG-1	HMG-2
Duerr et al. (13)	EL	32	2	10				0	0						
Nassberger et al. (14)	EL	24 ^c	3	54	80		57	3	3		3				
Hauschild et al. (15)	EL	ND	ND	72	24					15					
Kaneko et al. (16)	EL	15	3	6			0								
Schmitt et al. (17)	EL	120	ND	49						21					
Peen et al. (18)	EL	218	2	24									50		
Mulder et al. (19)	WB			67	51								22		
Broekloelofs et al. (20)	EL	252	3	67	49			0	0		4				
Kossa et al. (21)	EL	35	3	49	69			46	46	53	41				
Yang et al. (22)	EL	190	2	36	64		3				8				
Stoffel et al. (23)	EL	140	3	54	72	37									
Sobajima et al. (24)	EL	27	3	35				26			9				
Walmsley et al. (26)	EL	46	3	52	67	29		4			4				
Sobajima et al. (25)	EL	37	3	60	85			43			13			32	33
Roozendaal et al. (27)	WB			96	58	2					26	38	10		

^a Number of standard deviations above the mean of healthy controls that served as a cutoff point for a positive value.

^b β -Glu, β -glucuronidase; EL, ELISA; ND, no data available; WB, Western blot.

^c Colitis controls.

Table 2. Prevalence of autoantibodies to specific antigens in patients with CD.

Study	Method	Controls		Patients, n	Positive antibody test, %									
		n	SD ^a		ANCAs	BPI	β -Glu ^b	Cathepsin G	Elastase	Lysozyme	Lactoferrin	Catalase	α -Enolase	
Mayet et al. (28)	EL	84	3	60				38	3					
Schmitt et al. (17)	EL	120	ND	23						17				
Peen et al. (18)	EL	218	2	52	27							8		
Mulder et al. (19)	WB			35	40							17		
Broekloelofs et al. (20)	EL	252	3	35	40			0	0			8		
Kossa et al. (21)	EL	35	3	33	39			12	18	39		3		
Yang et al. (22)	EL	190	2	37	5		8					3		
Stoffel et al. (23)	EL	140	3	44	45	23								
Walmsley et al. (26)	EL	46	3			14		27				3		
Roozendaal et al. (27)	WB			112	19	5						11	26	18

^a Number of standard deviations above the mean of healthy controls that served as a cutoff point for a positive value.

^b β -Glu, β -glucuronidase; EL, ELISA; ND, no data available; WB, Western blot.

teases cathepsin G and elastase, the hydrolase β -glucuronidase, the iron-binding protein lactoferrin (80 kDa), and the natural antibiotic bactericidal permeability increasing (BPI) protein are located in the granules of the neutrophils (and monocytes). α -Enolase (47 kDa), which is involved in glycolysis, and catalase (60 kDa), which catalyzes the dissociation of hydrogen peroxide to water and oxygen, are cytoplasmic proteins. High-mobility group (HMG)-1 nonhistone chromosomal proteins and HMG-2 nonhistone chromosomal proteins (28–29 kDa) are distributed in the nuclei and cytoplasm of eukaryotic cells and act as transcription factors. HMG-1 stimulates transcription by modulating the structure of chromatin in vitro and in cultured cells. HMG-2 (29 kDa) is closely

related to cell proliferation. Antibodies to histone H1 have been suggested, but their prevalences have not been reported (29). Myeloperoxidase and proteinase-3, constituents of the azurophilic granules, are not autoantigens in IBD.

The various studies have revealed heterogeneous and conflicting results. Most studies have used ELISA to identify the antibodies, except for 2 studies in which Western blotting was used (see Tables 1–3). Many of the studies considered a sample positive if the absorbance value obtained by ELISA exceeded the mean of healthy control samples by >3 SD. Some studies used 2 SD instead of 3 SD (see Tables 1–3). This means that in the majority of the studies, the cutoff was chosen in a way such that positive results were found in only a small portion of the healthy control population. In one study, in which Western blotting was used to detect antibodies, anti-BPI, anti-elastase, anti-catalase, and anti- α -enolase antibodies were found in 0%, 0%, 10%, and 5% of healthy controls, respectively (27). A shortcoming of many studies is that the antibodies have been studied in well-defined CD or UC patients but not in diseased controls, i.e., non-IBD patients (e.g., infective diarrhea) who present with symptoms similar to the symptoms in IBD. This implies that data on the specificity of the antibodies studied are incomplete. One study reported anti-BPI and anti-cathepsin G antibodies in a substantial number ($\geq 20\%$) of infectious enteritis cases (26). Direct comparison of the sensitivities and specificities of the serologic markers described in the various studies was not possible because no ROC curves could be constructed based on the data provided in the different reports. Ideally, sensitivities should be compared at a fixed specificity and vice versa. Finally, it should be mentioned that no generally accepted method for detecting the various antibodies has been described and that no serum standards exist for the different antibodies listed in Tables 1–3. These are shortcomings that weaken the use of serology in patients in IBD and that also may explain the wide range of results observed among the studies reported. The differences

Table 3. Prevalence of autoantibodies to specific neutrophilic antigens in controls.

Group studied	Study		
	Walmsley et al. (26)	Yang et al. (22)	Roozendaal et al. (27)
Method	Infectious enteritis	Collagenous colitis	Healthy controls
Healthy controls	EL ^a	EL	WB
n	46	190	
SD ^b	3	2	
Patients, n	30	38	78
Positive antibody test, %			
ANCAs	23	0	
BPI	23		0
β -Glucuronidase		3	
Cathepsin G	20		
Elastase			0
Lysozyme			
Lactoferrin	7	0	
Catalase			10
α -Enolase			6

^a EL, ELISA; WB, Western blot.

^b Number of standard deviations above the mean of healthy controls that served as a cutoff point for a positive value.

between the studies could be attributable not only to variations in methodology and the choice of the controls for determination of the ELISA cutoff values, but also to differences in the patient population (diagnostic criteria, severity of disease, treatment). Most studies found that the (azurophilic) granule proteins of the neutrophil were not recognized by most of P-ANCAs in IBD patients. Multiple antibodies against different antigens were found in 1 patient, and antibodies to several antigens were also found in immunofluorescent-negative samples (30). This is particularly the case for the cytoplasmic autoantigens, which are not packed and concentrated in granules. For example, only 21% and 38% of IBD samples positive for antibodies to catalase and α -enolase, respectively, were positive for ANCA antibodies by indirect immunofluorescence (27).

Various target antigens of atypical P-ANCAs, such as catalase, enolase, histone H1, and HMG nonhistone chromosomal proteins, are not uniquely present in neutrophils or other myeloid cells, but are found in most higher eukaryote somatic cells. β -Glucuronidase is also found in bacteria. It is not clear why these antigens cause atypical P-ANCAs. Specific epitopes of these proteins recognized by atypical P-ANCAs may be unique or only immunoinaccessible in neutrophils.

Taken together, most studies support the conclusion that the neutrophil (azurophilic) granule components characterized to date are not UC-associated P-ANCA-specific antigens and that the main target antigen has not yet been identified. The idea that a nuclear antigen is the target of these antibodies has been suggested by several authors.

Vidrich et al. (31) reported loss of antigenic recognition after DNA digestion of neutrophils for UC P-ANCAs but not for P-ANCAs of primary sclerosing cholangitis and autoimmune hepatitis. The P-ANCA patterns in primary sclerosing cholangitis and autoimmune hepatitis convert to a C-ANCA staining pattern after DNase treatment. This suggests that the epitope recognized by the UC P-ANCA is a protein-DNA complex or that the presence of intact DNA is necessary for maintaining the integrity of the epitope. The atypical P-ANCA of UC and primary sclerosing cholangitis is specific for neutrophils, whereas that of autoimmune hepatitis reacts with both neutrophils and monocytes. Confocal microscopy revealed a nuclear reaction for 88% (22 of 25) of the sera, with 72% (18 of 25) showing the reaction localized to the inner side of the nuclear (membrane) periphery (32). Immunoelectron microscopy showed that the UC-associated P-ANCA reaction localized primarily over chromatin concentrated toward the nuclear periphery. The sera did not recognize double-stranded DNA. Sobajima et al. (24) identified HMG nonhistone chromosomal proteins as a possible target. Terjung et al. (33) described a 50-kDa protein (pI 6) that colocalized with proteins of the nuclear envelope of neutrophils. This protein was confined to myeloid cells and was recognized by >90% of atypical P-ANCAs in

individuals with IBD, primary sclerosing cholangitis, and autoimmune hepatitis. Terjung et al. (12) reported that the atypical P-ANCAs also show multiple intranuclear fluorescent foci, which likely correspond to infoldings of the nuclear envelope.

As long as the target antigen recognized by atypical P-ANCAs remains unidentified, sensitive and specific solid-phase assays cannot be developed, leaving immunofluorescence microscopy as the only widely available technique for the detection of these antibodies.

Joossens et al. (34) evaluated the interassay and interobserver variability in the detection of UC-associated ANCAs. In the interobserver study, the same assay was used by different readers in 4 geographically distinct laboratories [INOVA Diagnostics (San Diego, CA), University of Iowa Hospital (Iowa City, IA), Ospedale Mauriziano Umberto I (Torino, Italy), and University Hospital Leuven, (Leuven, Belgium)]. In the interobserver study, the authors found moderate to substantial agreement (κ -values between 0.4 and 0.65–0.8). The prevalence of ANCAs varied between 56% and 70%. In the intermethod study, ANCAs were assayed by 1 experienced reader using substrates from 4 different commercial sources (The Binding Site, Bio-Rad, INOVA, and Immunoconcepts). The prevalence of ANCAs varied between 18% and 68%, with κ -values <0.2, indicating poor agreement. Similar results were obtained by Sandborn et al. (35). The sensitivity for ANCA detection in 150 UC patients varied between 0% and 63% in 5 different laboratories (Prometheus, Oxford, Wuerzburg, Mayo, and Smith Kline Beecham).

The perinuclear nature of some ANCA reactions (e.g., antibodies to myeloperoxidase or elastase) is an artifact of the alcohol fixation of neutrophils, which causes positively charged cytoplasmic granular proteins to redistribute around the negatively charged nucleus. When neutrophils are fixed with non-alcohol-based reagents (e.g., paraformaldehyde or formalin), the perinuclear reaction obtained with either myeloperoxidase-P-ANCA or elastase-P-ANCA is abolished and converted to a cytoplasmic reaction pattern.

Published data about the reactivity of atypical P-ANCAs on formaldehyde-fixed neutrophils in IBD and autoimmune liver disorders are rare and controversial. Some investigators did not detect any fluorescence on formaldehyde-fixed neutrophils in most patients with IBD (34), whereas others reported a cytoplasmic (19, 30) or an atypical P-ANCA (33) formalin-positive reactivity in a high portion of the sera. Variations in the formaldehyde fixation techniques and the resolution of the immunofluorescence microscope used might be responsible for the controversial reports about the microscopic features of atypical P-ANCAs on formaldehyde-fixed neutrophils. Even use of the same technique in the same laboratory may lead to inconsistent results.

The serum dilution for the determination of P-ANCAs was 1:40 or 1:20 for most of the studies. Some studies were

done with the Prometheus UC Diagnostic system. This is a 3-step process that includes ELISA analysis, an indirect immunofluorescence assay, and DNase treatment. Samples were considered positive if a certain cutoff value on ELISA analysis was exceeded.

Anti-*Saccharomyces cerevisiae* Antibodies

Increased concentrations of antibodies to the baker's and brewer's yeast *Saccharomyces cerevisiae* (ASCAs) are found in patients with CD (36). Both IgG and IgA antibodies are formed. They have been demonstrated in 60%–70% of patients with CD, 10%–15% of patients with UC, and 0%–5% of control individuals (Table 4). The antibodies recognize carbohydrate epitopes in phosphopeptidomannan, a 200-kDa glycoprotein of the cell wall (37). The major epitope was identified as mannotetraose (38). The highest sensitivity and specificity of the serologic response was found with strain Su1 (38).

A comparative study revealed a wide range in sensitivities and specificities among 4 assays (3 commercially available assays and the assay developed in Lille, Centre Hospitalier Régional Universitaire, France), mainly as a consequence of the cutoff values chosen (39). The agreement among the assays was good (39).

In contrast to P-ANCAs, ASCAs do not seem to be autoantibodies but rather antibodies against bacterial or fungal species. The presence of ASCAs in patients with IBD may be the result of a response to either the antigens on *S. cerevisiae* itself or to an as yet unidentified antigen that cross-reacts with *S. cerevisiae* antigens.

Diagnostic Value of Autoantibodies to Neutrophils (Atypical P-ANCAs) and ASCAs in IBD

The prevalences of P-ANCAs and ASCAs in UC, CD, diseased controls, and healthy controls (35, 40–44) are summarized in Table 4. Atypical P-ANCAs are (50%–67%) found mainly in UC, but also in CD (6%–15%) and to a lesser extent in diseased controls (<11%). Atypical P-ANCAs are also found in autoimmune hepatitis and primary sclerosing cholangitis. The prevalence of ASCAs

is higher in CD (40%–60%), but they are also found in UC and in diseased controls (4%–14%). In healthy controls, the antibodies are present in <5%. The clinical value of ANCA or ASCA testing in diarrheal diseases is limited because of insufficient sensitivity (45) and imperfect specificity.

The combination of atypical P-ANCAs and ASCAs may be useful in the differential diagnosis of UC and CD in patients with IBD. A survey of studies (35, 40–42, 46) that combined the 2 markers to distinguish UC from CD is given in Table 5. The CD-associated pattern was ASCA⁺/P-ANCA[−], whereas the UC-associated pattern was ASCA[−]/P-ANCA⁺. The combined evaluation of ANCAs and ASCAs had a higher specificity (>90% in most studies and >80% in all studies) to differentiate CD from UC than the separate use of either ANCAs or ASCAs. The increased specificity, however, was associated with decreased sensitivity.

In addition to sensitivity and specificity, positive predictive values are provided in some studies. For example, in patients with IBD, the positive predictive value of the combination of a positive ASCA test with a negative P-ANCA test for UC has been reported to be 92.5% by Quinton et al. (41), 88% by Peeters et al. (40), and 93% by Koutrobakis et al. (42). The positive predictive values of the combination of a negative ASCA test with a positive P-ANCA test for UC has been reported to be 96% by Quinton et al. (41), 95% by Peeters et al. (40), and 77% by Koutrobakis et al. (42). The positive predictive value places test specificity in the context of disease prevalence and indicates what percentage of patients with positive test results actually have the disease. In these studies, however, the authors artificially controlled the prevalence of the disease by focused recruiting of patients and healthy and diseased controls. The prevalence of disease in a clinical situation is different from the prevalence in most research studies.

The likelihood ratio is less likely to change with the prevalence of the disorder and incorporates both the sensitivity and specificity of the test. It provides a direct

Table 4. Prevalence of ASCAs and P-ANCAs in CD, UC, diseased controls, and healthy controls.

Antibodies	Study	n ^a	Positive for antibodies, %			
			CD	UC	Diseased controls	Healthy controls
ASCAs	Peeters et al. (40)	147/407/74/157	60	14	11	3.2
	Koutrobakis et al. (42)	97/56/0/150	39	11		0.7
	Quinton et al. (41)	101/100/27/163	61	12	11	0.6
	Ruemmele et al. (43)	36/131/78/0	55	5	5	
	Hoffenberg et al. (44)	25/20/74/0	60		4	
P-ANCAs	Peeters et al. (40)	147/407/74/157	6	50	8	2.5
	Koutrobakis et al. (42)	97/56/0/150	16	67		0
	Quinton et al. (41)	101/100/27/163	15	66	7.5	0.6
	Ruemmele et al. (43)	36/131/78/0	13	57	0	
	Hoffenberg et al. (44)	25/20/74/0		60	11	

^a UC/CD/diseased controls/healthy controls.

Table 5. Ability of ANCA and ASCAs to differentiate UC from CD in patients with IBD.

	Study				
	Quinton et al. (41)	Linskens et al. (46)	Koutroubakis et al. (42)	Sandborn et al. (35)	Peeters et al. (40)
UC, n	101	51	97	83	147
CD, n	100	50	56	79	407
Sensitivity, %					
ASCA ⁺ (CD)	61	72	39	44	60
P-ANCA ⁺ (UC)	65	63	67	63	50
ASCA ⁺ /P-ANCA ⁻ (CD)	49	64	30	38	56
P-ANCA ⁺ /ASCA ⁻ (UC)	57	51	58	55	44
Specificity, %					
ASCA ⁺ (CD)	88	82	89	87	86
P-ANCA ⁺ (UC)	85	86	84	75	94
ASCA ⁺ /P-ANCA ⁻ (CD)	97	94	97	94	92
P-ANCA ⁺ /ASCA ⁻ (UC)	97	94	88	81	98
Positive likelihood ratio					
ASCA ⁺ (CD)	5	4	3.5	3.3	4.2
P-ANCA ⁺ (UC)	4.3	4.5	4.1	2.5	8.3
ASCA ⁺ /P-ANCA ⁻ (CD)	16	11	10	6.3	7
P-ANCA ⁺ /ASCA ⁻ (UC)	19	8.5	4.8	2.9	22
Negative likelihood ratio					
ASCA ⁺ (CD)	0.44	0.3	0.68	0.64	0.46
P-ANCA ⁺ (UC)	0.41	0.43	0.39	0.49	0.53

estimate of how much a test result will change the odds of having a disease (47, 48). The likelihood ratio for a positive result tells how much the odds of the disease increase when a test is positive; conversely, the likelihood ratio for a negative result tells how much the odds of the disease decrease when a test is negative. The likelihood ratio can be combined with information about the prevalence of the disease and the characteristics of the patient group and the patient to determine the posttest odds of disease in a particular patient. Thus, the likelihood ratios allow the clinician to estimate whether there will be a significant change in the pretest to posttest probability of a disease as a result of obtaining the test (47, 48). A likelihood ratio of 1 implies that the test is of no clinical value. Likelihood ratios >10 or <0.1 indicate very large, clinically important differences in pretest-posttest probability. Likelihood ratios between 5 and 10 or between 0.1 to 0.2 often lead to more modest, but still substantial, differences in pretest-posttest probability. Ratios from 2 to 5 or from 0.5 to 0.2 generate small differences that may be relevant in certain clinical settings. Likelihood ratios between 1 and 2 or 0.5 and 1 generate very small differences that are seldom clinically important (47, 48).

I have calculated the likelihood ratios of P-ANCA and ASCA results (a) to distinguish IBD from non-IBD and (b) to distinguish CD from UC in IBD patients. The likelihood ratios to discriminate IBD from non-IBD were calculated based on data obtained from the study of Peeters et al. (40). The likelihood ratio of a positive result for ASCAs, P-ANCA, ASCA⁺/P-ANCA⁻, and ASCA⁻/P-ANCA⁺ to distinguish patients with IBD (n = 554) from controls (n = 231) was, respectively, 6.6, 10, 9.3, and 14.6, and the

likelihood ratio of a negative result was 0.43, 0.52, 0.46, and 0.57, respectively. This indicates that in the context of distinguishing IBD patients from non-IBD individuals, a positive test result for ASCAs, P-ANCA, ASCA⁺/P-ANCA⁻, or ASCA⁻/P-ANCA⁺ substantially affects pretest-posttest probability, whereas a negative test result insignificantly affects pretest-posttest probability. The likelihood ratios of a positive result for atypical P-ANCA and for ASCAs to differentiate between UC and CD in patients with IBD are shown in Table 5 and was between 2 and 5 in all studies, indicating that these assays affect pretest-posttest probability to a small degree. The likelihood ratio of a negative test result for atypical P-ANCA and ASCAs was between 0.3 and 0.7 in all conditions, signifying poor clinical importance of a negative test result for differentiating CD from UC. By contrast, the combined evaluation of atypical P-ANCA and ASCAs had a positive likelihood ratio >5 in almost all studies and >10 in one-half of the studies (see Table 5). This implies that the combined use of atypical P-ANCA and ASCAs substantially affects pretest-posttest probability in distinguishing UC from CD in patients with IBD.

Thus, the combined use of ASCA and P-ANCA results could be an addition to conventional techniques (the patient's history, radiologic examination, endoscopy, and biopsy) in the differential diagnosis between CD and UC. It should be mentioned, however, that a high percentage of CD patients with pure colonic disease and UC-like colitis have been reported positive for P-ANCA (49), thereby constraining the sensitivity of the ASCA⁺/P-ANCA⁻ combination as a specific marker for CD.

Uses of Test Results for Autoantibodies to Neutrophils (Atypical P-ANCAs) and ASCAs

INDETERMINATE COLITIS

Serologic evaluation of ANCAs and ASCAs could be of help in patients with indeterminate colitis. In these patients, early knowledge of the exact diagnosis could be of clinical importance with regard to therapeutic decisions and prognosis. In a multicenter prospective study, 97 patients with indeterminate colitis were analyzed for ANCAs and ASCAs (50). After a 1-year follow-up, a definite diagnosis was reached in 31 of the 97 patients. The combination ASCA⁺/ANCA⁻ predicted CD in 80% of IC patients (sensitivity, 67%; specificity, 78%; positive likelihood ratio, 3), whereas ASCA⁻/ANCA⁺ was predictive for UC in 64% of the patients (sensitivity, 78%; specificity, 67%; positive likelihood ratio, 2.3). ASCA⁻/ANCA⁺ patients who did not progress to UC all developed UC-like CD. A remarkable finding in this study was that 48.5% of the patients did not have antibodies to either ASCAs or ANCAs and that these seronegative patients remained indeterminate after a mean duration of 9.9 years (50).

AS TOOLS FOR DISEASE MONITORING

There is no relationship between the presence or titer of ANCAs and UC activity (49, 51). The ANCA titer remains positive after colectomy (51). In addition, the presence of ASCAs is stable over time and is independent of CD activity and duration (49, 52). ASCA titers most often remain stable after treatment (52). Hence, serial measurement of ANCA and ASCA titers in IBD is not useful for follow-up of disease activity and prediction of relapses.

The authors of one study have suggested that the presence of ANCAs may correlate with chronic pouchitis after ileal pouch-anal anastomosis (53), but this has not confirmed in other studies (51, 54, 55).

ASSOCIATION WITH CLINICAL PHENOTYPES

Atypical P-ANCAs are not associated with clinical characteristics such as age of onset, need for surgery, location of the disease, or familial history (40). It has been suggested that in patients with CD, the presence of atypical P-ANCAs in serum characterizes a UC-like clinical phenotype (49). Patients with "UC-like CD" have endoscopically and/or histopathologically documented left-sided colitis and symptoms of left-sided colonic inflammation, clinically reflected by rectal bleeding and mucus discharge, urgency, and treatment with topical agents. In the study by Vasiliauskas et al. (49), the number of patients meeting the criteria of UC-like phenotype was 18 of 18 (100%) in the P-ANCA-positive CD subgroup, 9 of 20 (45%) in the C-ANCA subgroup, and 12 of 31 (39%) in the ANCA-negative CD subgroup.

On the other hand, ASCAs have been associated with several CD clinical phenotypes. ASCAs have been associated with stricturing as well as penetrating (56–59) disease behavior and with ileal disease (small-bowel

rather than colonic disease) (40, 41, 52, 57–59). For example, Quinton et al. (41) reported that 70% of CD patients with small bowel involvement (with or without colonic disease) had ASCA antibodies vs 46% of the patients with pure colonic disease, and Walker et al. (57) reported that 68% of patients with CD involving the ileum had ASCAs vs 38% of patients with colonic disease. Mow et al. (59) reported that 71.7% of CD patients with fibrostenosing disease had ASCAs vs 36.1% of patients without fibrostenosing disease and that 50.9% of patients with internal perforating disease had ASCAs vs 27.1% of patients without internal perforating disease. ASCAs have been associated with young age at diagnosis in several studies (40, 41, 52, 58) but not in others (57).

RESPONSE TO MEDICAL THERAPY

ANCA positivity has been reported to be associated with resistance to treatment of left-sided UC (60). Sanborn et al. (60) reported that 90% of patients with refractory left-sided colitis were ANCA-positive vs 62% of patients with treatment-responsive UC ($P = 0.03$). ASCAs and P-ANCAs were not associated with response to anti-tumor necrosis factor- α therapy (61). A trend toward poor response to anti-tumor necrosis factor- α therapy in CD patients carrying the ANCA⁺/ASCA⁻ combination has been suggested (61). This was, however, not statistically significant and needs to be confirmed.

AS SUBCLINICAL MARKERS

The strongest risk factor for IBD is having a relative with the disease. The occurrence of antibodies in unaffected family members could reflect a genetic and/or environmental factor predisposing to disease. In addition, the presence of antibodies could also be indicative of pre-symptomatic disease. Several groups have studied whether P-ANCAs and ASCAs are subclinical markers of IBD in families.

Some studies showed that 16%–30% of healthy first-degree relatives of UC patients were P-ANCA-positive (62, 63), whereas other studies were unable to find P-ANCAs in first-degree relatives (64–66).

Several studies have found an increased prevalence of ASCAs in unaffected first-degree relatives of patients with CD. Sendid et al. (67) detected ASCAs in 35 of 51 (69%) patients with CD and in 13 of 66 (20%) healthy relatives vs 1 of 163 healthy controls. In that study, the health status of the healthy relatives was assessed by an interviewer practitioner who visited the families. None of the healthy relatives had a previous history or symptoms of IBD. Seibold et al. (68) found ASCAs in 25% of 193 healthy first-degree relatives. Sutton et al. (69) reported familial aggregation of ASCA concentrations for affected relatives. Vermeire et al. (52) found that ASCA prevalence was the same in both sporadic and familial CD. Within pure CD families, ASCAs were present in 54% of CD patients with 2 family members affected vs 74.7% in CD patients with 3 or more family members affected (52).

There was no concordance of ASCA reactivity in marital pairs. These data support the suggestion that ASCAs reflect the familial load of the disease. Whether the presence of ASCAs is a familial trait attributable to a genetic factor or to an increased childhood environmental exposure that predisposes to the disease is unknown. A recent study in twins suggested that ASCAs are a marker of a response to an environmental antigen and that genes other than CARD15 determine the extent of the response (58).

Israeli et al. (70) demonstrated that ASCAs and P-ANCAs may predict development of IBD years before the disease is clinically diagnosed. ASCAs were present in 10 of 32 (31.3%) CD patients before clinical diagnosis compared with 0 of 95 (0%) controls ($P < 0.001$). ASCA test results were positive in 54.5% of patients after diagnosis of CD. The mean interval between ASCA detection and diagnosis was 38 months. P-ANCAs were present in 2 of 8 (25%) patients with available sera before the diagnosis of UC. None of the 24 matched controls were positive ($P = 0.014$).

Pancreatic Antibodies

Antibodies against exocrine pancreas have been described in patients with CD, and have been reported to be specific (not present in nondiseased individuals), albeit at a low prevalence (~30%) (71–73). The antigen has not been elucidated, and the antibodies are detected by indirect immunofluorescence. Joossens et al. (74) found pancreatic antibodies not only in CD patients but also, in low titers, in UC patients. The antibodies were also found in first-degree relatives of IBD patients, a finding that had not been observed in earlier studies (65, 75).

New Serologic Markers for IBD

Screening lysates of cultures of colonic bacteria with a monoclonal P-ANCA antibody revealed that *Escherichia coli* outer membrane porin (OmpC) is an antigen in IBD (76). Landers et al. (77) reported anti-OmpC antibodies in 55% of CD patients. Patients with anti-OmpC antibodies were more likely to have internal perforating CD (59). In children and young adults, anti-OmpC antibodies (IgG and IgA) were found in 24% of CD patients, 11% of UC patients, and 5% of controls (78).

Microbial sequence I2 was identified as a homolog of the tetR bacterial transcription factor family, which is frequently present in CD colonic lesions (43%) but not in other colonic specimens (79). This sequence has been shown to be associated with *Pseudomonas fluorescens* (80). IgA anti-I2 antibodies were found in 54% of CD patients, 10% of UC patients, 19% of patients with other inflammatory enteric diseases (infectious colitis, radiation-associated proctitis, *Shigella* colitis, eosinophilic colitis, and collagenous colitis), and 4% of healthy controls (79). Patients with anti-I2 antibodies were more likely to have fibrostenosing disease and require surgery (59).

The flagellin CBir1 has been identified as a dominant

antigen capable of inducing colitis in C3H/HeJBir mice and eliciting IgG antibody responses in a subpopulation (~50%) of patients with CD (81, 82). Anti-CBir1 expression is independent of other antibody responses, is associated with small-bowel, internal-penetrating, and fibrostenosing disease features, and defines a subgroup of CD patients not previously recognized by other serologic responses (82). Among the population of CD patients positive for P-ANCAs but who do not react to other known antigens, 40%–44% are positive for anti-CBir1 antibodies, whereas anti-CBir1 antibodies have been found in only 4% of P-ANCA⁺ UC patients (82).

There is evidence that the number and magnitude of immune responses to different microbial antigens (ASCA, OmpC, and I2) in a given patient are associated with the severity of the disease course; i.e., the greater the number of responses and the greater their magnitude, the more severe the disease course (fibrostenosis, internal perforating disease, and the need for small-bowel surgery) (59, 82). Patients positive for I2, OmpC, and ASCA were more likely to undergo small-bowel surgery (72%) than were patients without reactivity (23%) (59). These findings must be confirmed in future studies evaluating the association between the presence of antibodies to microbial antigens and the development of strictures and perforations and subsequent need for surgery. If such studies can confirm that antibodies can identify patients likely to undergo a severe and problematic disease course, then the determination of antibodies could be helpful in clinical practice.

Conclusions

Atypical P-ANCAs and ASCAs are markers for UC and CD, respectively. Their role as diagnostic serologic markers for IBD appears to be limited, however, mainly because of their lower sensitivity. A positive test result for either P-ANCAs or ASCAs modestly influences pretest–posttest probability in distinguishing IBD from non-IBD, and a negative test result has no clinical value. The combined use of atypical P-ANCA and ASCA test results substantially affects pretest–posttest probability in distinguishing UC from CD in patients with IBD. The P-ANCA⁺/ASCA[−] combination is specific for UC, whereas the ASCA⁺/P-ANCA[−] combination is specific for CD. This may be of help in patients in whom distinction between CD or UC is not obvious with the classic diagnostic tools (patient history, radiologic examination, endoscopy, and biopsy). The discriminative value of ASCAs and P-ANCAs to predict definitive diagnosis (CD or UC) in patients with indeterminate colitis is modest. Almost 50% of these patients do not develop ASCA or P-ANCA antibodies. Future studies should unravel whether this seronegative subgroup of patients represents a separate clinical entity.

Serial measurement of P-ANCAs and ASCAs is not useful. Titers of both antibodies are stable over time and do not correlate with disease activity. ASCA positivity is

correlated with ileal involvement of CD disease and stricturing as well as with penetrating disease behavior.

ASCAs are detectable in 20%–25% of first-degree relatives of patients with CD, but whether these antibodies are markers of future disease has yet to be determined.

The assays that detect atypical P-ANCAs and ASCAs lack standardization, which leads to large interlaboratory variation. Efforts should be undertaken to harmonize these assays, and future research should aim to identify the main autoantigens targeted by atypical P-ANCAs.

Pancreatic antibodies are specific markers for IBD. Their sensitivity, however, is limited (30%).

New microbial target antigens (OmpC, I2, and the flagellin CBir1) have been described in patients with CD. There is evidence that the number and magnitude of immune responses to different microbial antigens are associated with the severity of the disease course. This should be confirmed by additional studies.

Upcoming studies should further explore the potential to cluster patients in more homogeneous subgroups based on antibody responses. Correlating serologic markers with genotypes and clinical phenotypes should enhance our understanding of the pathophysiology of IBD. Hopefully this will lead to the introduction of new and accurate tools for diagnosis, stratification, and follow-up of patients with IBD.

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Anti-lactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangitis, and Crohn's disease

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Abstract

Fifty two serum samples from patients with Crohn's disease, 24 from patients with ulcerative colitis, and 12 from patients with primary sclerosing cholangitis were analysed for the presence of anti-neutrophil cytoplasm antibodies (ANCA) of IgG and IgA class by means of enzyme linked immunosorbent assays using lactoferrin, myeloperoxidase, and antigen extracted from azurophil granules, 'α antigen' (that is, an antigen preparation containing proteinase 3) as substrates. A high frequency of positive tests for IgG anti-lactoferrin antibodies was found in sera from patients with ulcerative colitis (50%) and primary sclerosing cholangitis (50%). In Crohn's disease only 4 of 52 (8%) sera had anti-lactoferrin antibodies - in all four instances the sera belonged to patients with disease involving the colon. All patients with sclerosing cholangitis and positive tests for anti-lactoferrin had ulcerative colitis. Low levels of IgG antibodies against myeloperoxidase or α antigen were also found occasionally in sera from patients with ulcerative colitis and primary sclerosing cholangitis. IgA antibodies directed against lactoferrin and α antigen (but not myeloperoxidase) were seen in all three conditions.

(Gut 1993; 34: 56-62)

Since the description of anti-neutrophil cytoplasm antibodies (ANCA) in active Wegener's granulomatosis,¹ the interest in anti-granulocyte antibodies has increased vastly. ANCA is now recognised as a family of autoantibodies directed against cytoplasmic antigens, mainly lysosomal enzymes, in polymorphonuclear neutrophil leukocytes (PMNL).² In Wegener's granulomatosis, ANCA are typically directed against proteinase 3, a serine proteinase located in azurophilic granules of human PMNL.³ Anti-proteinase 3 antibodies may also occur in isolated, rapidly progressive glomerulonephritis and occasionally in systemic vasculitic conditions other than Wegener's granulomatosis.² Antibodies directed against other lysosomal enzymes in PMNL azurophilic granules, for example myeloperoxidase and elastase, also occur in primary systemic vasculitic diseases and rapidly progressive glomerulonephritis.^{3,4} After ethanol fixation of PMNL, proteinase 3 remains within the cytoplasmic granules, whereas myeloperoxidase and elastase become extracted and locate close to or on the cell nucleus.^{2,4} At indirect immunofluorescent microscopical determination of ANCA, anti-proteinase 3 antibodies produce a typical pancytoplasmatic granular

staining pattern (C-ANCA), whereas anti-myeloperoxidase and anti-elastase antibodies give rise to a blurry perinuclear staining pattern (P-ANCA) or staining of the PMNL nuclei (granulocyte specific anti-nuclear antibodies, GS-ANA).^{2,4,5} A detergent extract of isolated azurophil granules (α antigen) can be used for the detection of anti-proteinase 3 antibodies (C-ANCA) by means of enzyme linked immunosorbent assay (ELISA) but does not allow detection of anti-myeloperoxidase.^{4,6} Also, antibodies against lactoferrin, an iron binding protein residing in specific granules of PMNL,⁷ produce a P-ANCA pattern owing to perinuclear/nuclear localisation of lactoferrin after ethanol fixation.⁸ Apart from the artifactual GS-ANA staining pattern caused by antibodies directed against nucleophilic cytoplasmic antigens, it is possible that true GS-ANAs also exist.⁹

Apart from the occurrence in primary systemic vasculitides and rapidly progressive glomerulonephritis, P-ANCA/GS-ANA may be seen in other disease states, for example rheumatoid arthritis without signs of vasculitis,¹⁰ inflammatory bowel disease, and primary sclerosing cholangitis.¹¹⁻¹⁵ Recent evidence favours the idea that inflammatory bowel disease may be caused by mesenteric vasculitis.¹⁶⁻¹⁸

In a preliminary study of 16 frozen sera from patients with Crohn's disease we found low levels of anti-α antigen antibodies, anti-myeloperoxidase antibodies, and anti-lactoferrin antibodies in some sera.¹⁹ The present study was done to extend these observations and to include sera from patients with ulcerative colitis and primary sclerosing cholangitis.

Patients and methods

CROHN'S DISEASE

Fifty two patients, 27 men aged 21-71 years (mean 48 years) and 25 women aged 17-55 years (mean 38 years) were enrolled in the study. The mean duration of the disease was 17 years (range 4-33 years). Eight patients had disease limited to the colon and/or rectum, 19 patients had disease in the small bowel alone, and 25 patients had disease manifestations in both small bowel and colon/rectum. Forty patients had been operated on, 14 of whom had been subjected to only ileocecal resection. Three patients had ileorectal anastomosis, and six patients had ileostomy after proctocolectomy.

ULCERATIVE COLITIS

Twenty four patients, 11 men aged 19-65 years

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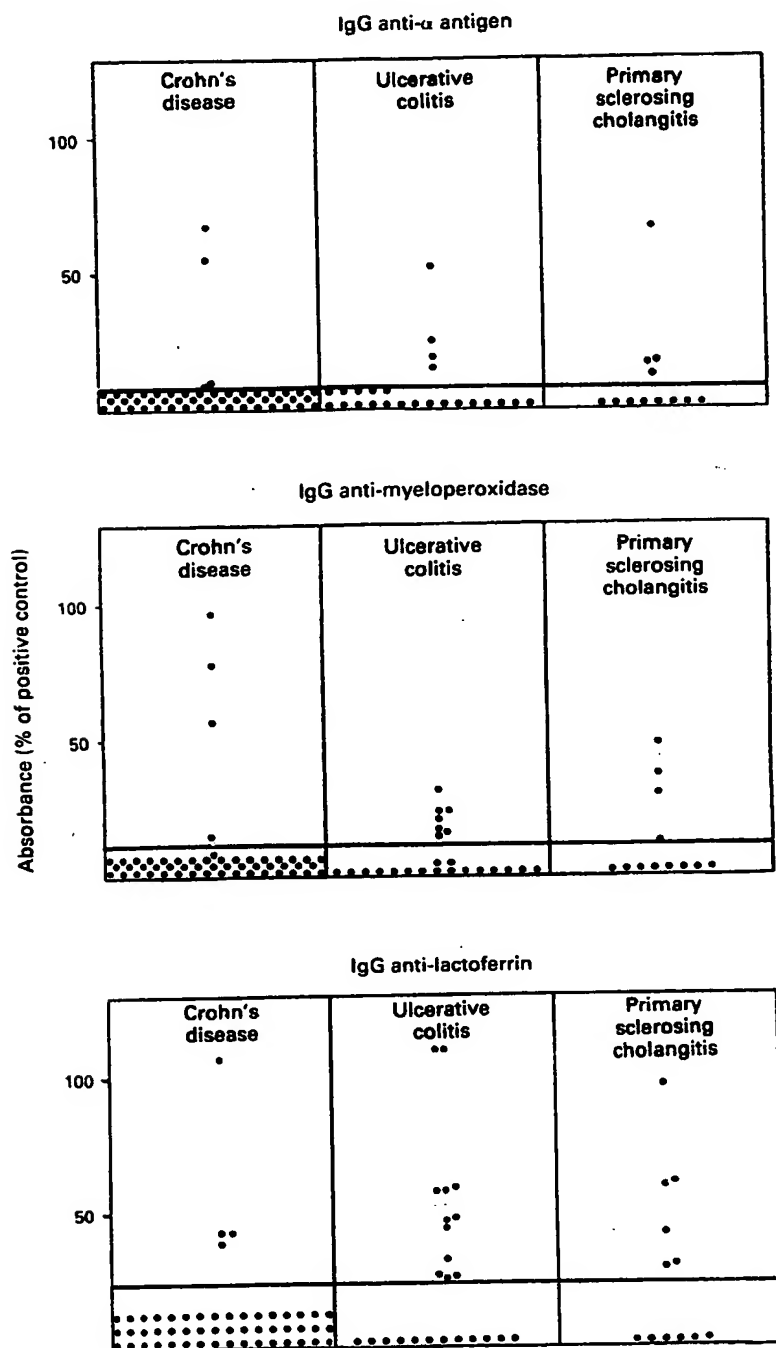


Figure 1: Diagram to illustrate the ELISA results of IgG autoantibody directed against α antigen, myeloperoxidase, and lactoferrin in sera from patients with Crohn's disease ($n=52$), ulcerative colitis ($n=24$), and primary sclerosing cholangitis ($n=12$). The lower limit for positive ELISA (that is, $+2$ SD of the mean value of 218 normal reference sera) is indicated.

(mean 46 years), and 13 women aged 20–68 years (mean 42 years) participated in the study. Nineteen patients had left sided colitis and five patients had more extensive or total colitis. Fourteen of the patients had active disease – that is at least five loose stools with visible blood (all had visible inflammation at endoscopy) – and the remaining 10 patients were in clinical remission.

PRIMARY SCLEROSING CHOLANGITIS

Twelve patients, seven men aged 20–59 years (mean 38 years) and five women aged 35–63 years (mean 48 years), took part in the study. The

diagnosis had been made by endoscopic retrograde cholangiography 3 years (range 1–6 years) before the study. Two patients had only intrahepatic cholangitis and 10 had both extra- and intrahepatic cholangitis. In four patients cirrhosis of the liver had been diagnosed at histopathological examination of liver biopsies. One patient had undergone liver transplantation 6 years earlier. Nine of the 12 patients had also suffered from ulcerative colitis for 8 years (range 0–21 years) and one had non-specific colitis. In the two remaining patients no accompanying disease had been diagnosed. None of the patients had Crohn's disease.

One of the patients originally participating in the study had to be excluded as she proved to have suffered from acute *Campylobacter jejuni* colitis.

ANTIGEN PREPARATIONS

Azurophil granules were prepared from isolated normal peripheral granulocytes after low pressure homogenisation of the cells and centrifugation of the homogenate in a Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient as described elsewhere.⁵ The granules were lysed with 0.01% (final concentration) Triton X-100 (Merck, Darmstadt, Germany).⁵ The extract thus achieved (α antigen) was diluted in carbonate-bicarbonate buffer pH 9.5 and used as substrate for ELISA.⁵

Lyophilised preparations of myeloperoxidase (Calbiochem, La Jolla, CA, USA), human milk lactoferrin (Sigma Chemical Co, St Louis, MO, USA), and bovine milk lactoferrin (Sigma) were dissolved in carbonate-bicarbonate buffer (10 μ g antigen/ml) and used for ELISA.

ELISA

High binding plastic microtitre plates (Nunc Immunoplate, Roskilde, Denmark) were coated with α antigen, myeloperoxidase, or human lactoferrin solutions by incubation at 4°C for 12 hours. After washing with buffer (phosphate buffered saline, PBS, with 0.05% Tween 20), serum samples (diluted 1:10 in PBS-Tween) were applied for 30 minutes at room temperature. Normal human serum diluted 1:10 served as a blank. The microtitre plates were washed thoroughly with PBS-Tween and incubated for another 30 minutes with alkaline-phosphatase (ALP) conjugated rabbit anti-human γ chain or rabbit anti-human α chain antisera (Dako, Glostrup, Denmark) diluted 1:400 in PBS-Tween. After washing, the substrate buffer was applied and the optical density (OD) read at 405 nm when the positive reference samples had reached OD 1.0. OD values exceeding 2 SDs of the reference material from healthy blood donors ($n=218$ for IgG tests; $n=211$ for IgA tests) were considered positive. The IgG autoantibody analyses were performed on all sera from patients with Crohn's disease ($n=52$), ulcerative colitis ($n=24$), and primary sclerosing cholangitis ($n=12$). IgA autoantibody tests were done on sera from 51 patients with Crohn's disease, 21 ulcerative colitis sera, and 11 sera from patients with primary sclerosing cholangitis.

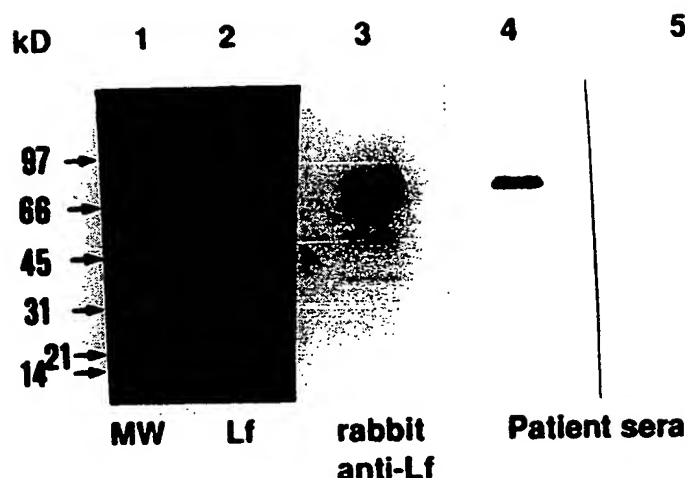


Figure 2: Sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) electrophoresis of the human milk lactoferrin preparation (lane 1) showed only material of molecular weight about 80 kD when stained with Coomassie blue. Lane 2 shows molecular weight markers for comparison. Immunoblotting, using polyclonal rabbit anti-human leukocyte lactoferrin, showed slight reaction with antigens of lower molecular weight, apart from the strong reaction with the 80 kD lactoferrin band (lane 3). Patient sera positive for IgG anti-human milk lactoferrin, as tested by ELISA, however, reacted only with lactoferrin.

IgG anti-bovine lactoferrin antibodies were measured essentially as described above, except that no negative or positive serum references were used. OD values were read after 30 minutes incubation with substrate buffer. Eleven positive and 10 negative sera in the test for IgG anti-human lactoferrin were selected for this analysis.

RABBIT ANTI-LACTOFERRIN ANTISERA

Serum containing polyclonal rabbit anti-human leukocyte lactoferrin was the kind gift of Dr Johan Richter, Department of Internal Medicine, University Hospital, Lund, Sweden. To produce anti-human milk lactoferrin antibodies, rabbits were immunised by subcutaneous injections of 2 mg human milk lactoferrin (that is, the same antigen as that used for the ELISA and western blot assays) together with Freund's incomplete adjuvant (Sigma) on three occasions with 4 week intervals. The reason for not using Freund's complete adjuvant was to avoid immunisation against hsp-65, because of its immunological cross reaction with human lactoferrin.²⁰ Two weeks after the second booster dose, the animals were anaesthetised with barbiturate, and exsanguinated by heart puncture. Serum was pooled. Anti-lactoferrin activity of the serum was confirmed by agarose double radial immunodiffusion against human milk lactoferrin.

WESTERN BLOTTING

Lactoferrin (1 µg per lane) was electrophoresed in 5–20% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore Continental Water Systems, Bedford, MA, USA). Blocking was done with 5% bovine serum albumin (Sigma) in PBS. Anti-lactoferrin positive patient sera (as judged by ELISA) were diluted 1:20 or 1:200 in PBS-Tween with 1% bovine serum albumin, and applied for 1 hour.

The rabbit anti-human leukocyte lactoferrin served as a reference. ALP conjugated anti-human IgG and anti-rabbit IgG (Dako) were used as secondary antibodies.

INDIRECT IMMUNOFLUORESCENCE (IIF) MICROSCOPY

To study the distribution of lactoferrin in ethanol fixed granulocytes cytocentrifuged onto microscope slides, the slides were incubated with rabbit anti-human milk lactoferrin in a moist chamber for 30 minutes. After washing with PBS and incubation for another 30 minutes with sheep fluorescein isothiocyanate (FITC) conjugated anti-rabbit Ig (Wellcome Diagnostics, Temple Hill, Dartford, UK), the slides were again washed with PBS, mounted with PBS-glycerin, and inspected under a fluorescence microscope with a mercury lamp (HBO 50) epillumination and filters for FITC activation/emission.

STATISTICS

Differences in ELISA results between the control sera and the patient sera were evaluated by the χ^2 test, and divided into four groups: $p \geq 0.05$ = not significant (NS); $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Results

The frequencies of IgG antibodies directed against α antigen, myeloperoxidase, and lactoferrin are shown in Figure 1.

In Crohn's disease 4 of 52 sera had IgG anti- α antigen, which is not statistically different from the control group, although two of the sera had remarkably high antibody levels. In ulcerative colitis 4 of 24 of the sera contained IgG anti- α antigen antibodies ($p < 0.05$), and in primary sclerosing cholangitis 4 of 12 sera ($p < 0.001$) were positive.

IgG anti-myeloperoxidase antibodies occurred in 3 of 52 of the sera taken from patients with Crohn's disease (not statistically different from

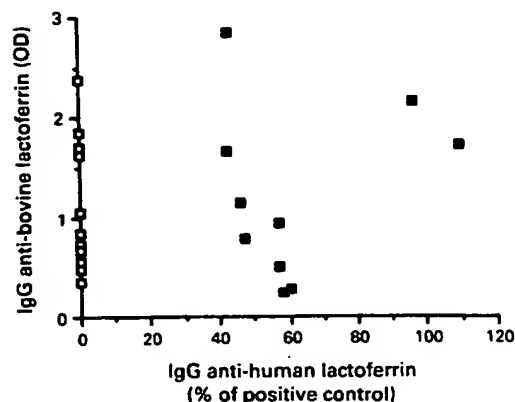


Figure 3: Diagram to illustrate the lack of correlation between ELISA results for IgG anti-human lactoferrin autoantibodies and IgG antibodies directed against bovine milk lactoferrin. Ten patient sera positive in the ELISA for IgG anti-human lactoferrin (■) and 12 randomly selected normal reference sera (○) negative in the IgG anti-human lactoferrin test were analysed.

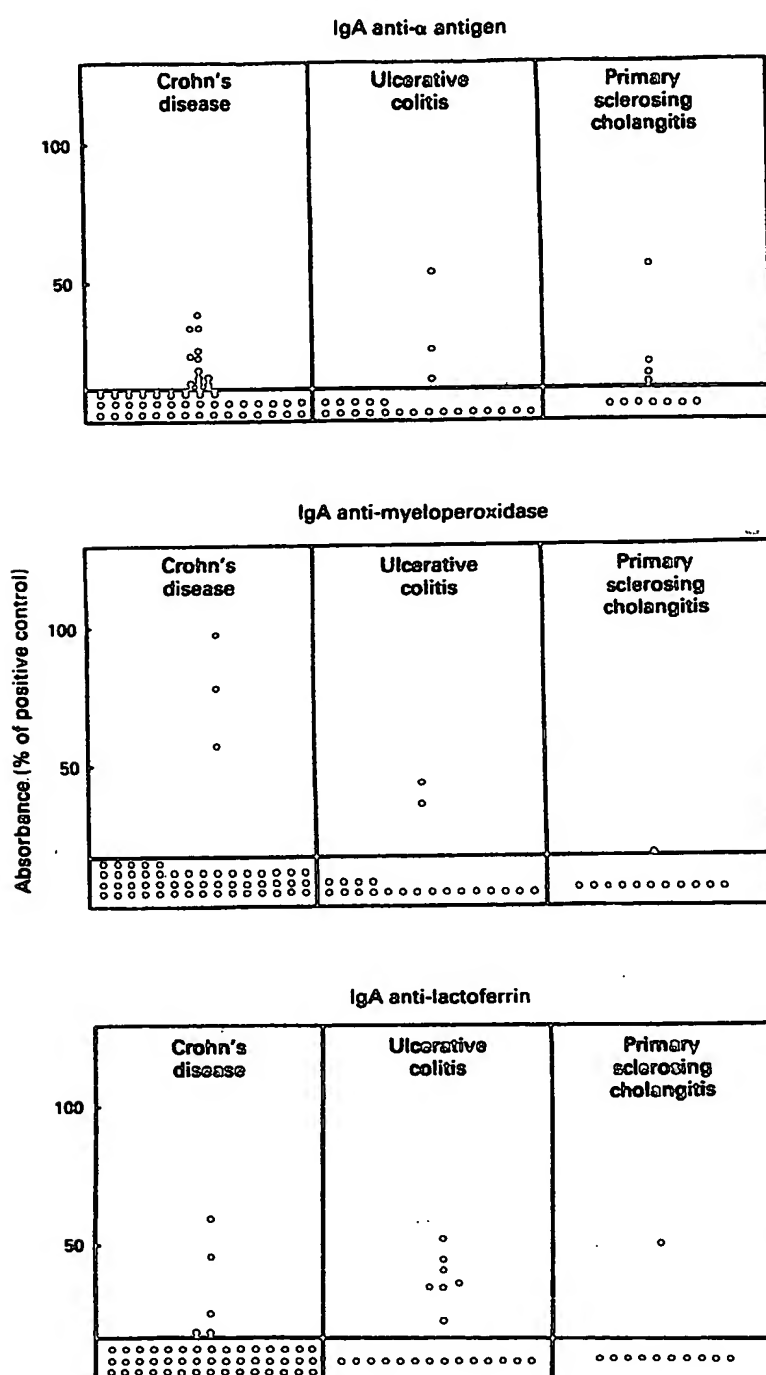


Figure 4: Results of the ELISA tests for IgA autoantibodies directed against α antigen, myeloperoxidase, and lactoferrin. Fifty one sera from patients with Crohn's disease, 21 sera from patients with ulcerative colitis, and 11 sera from patients with primary sclerosing cholangitis were analysed. The cut of levels (that is, values exceeding 2 SD of the mean of a reference material of 211 normal sera) are indicated.

the control group), and in 7 of 24 of the ulcerative colitis sera ($p < 0.001$). Four of 12 sera from patients with primary sclerosing cholangitis were positive ($p < 0.001$).

Four of 52 (8%) of the sera from patients with Crohn's disease contained IgG anti-lactoferrin antibodies (not significantly different from the control group). All of these sera proved to come from patients with Crohn colitis. However, colitis also occurred in 33 of 48 (69%) without IgG anti-lactoferrin antibodies. Both in ulcerative colitis and primary sclerosing cholangitis,

IgG anti-lactoferrin antibodies were found in 50% of the sera ($p < 0.001$ in both instances). All of the anti-lactoferrin positive cholangitis sera belonged to patients suffering from concomitant ulcerative colitis – that is, 6 of 9 (67%) sera from patients with sclerosing cholangitis and concomitant ulcerative colitis contained IgG anti-lactoferrin antibodies. Two of the sera from patients with primary sclerosing cholangitis and one serum from a patient with ulcerative colitis were positive in all three ELISA tests. However, in all three instances the ELISA tests gave quite different OD values in the different assays, indicating true positive ELISA test results.

Positive anti-lactoferrin ELISA results were blocked by preincubation of the lactoferrin-coated microtitre plates with rabbit IgG anti-lactoferrin (not illustrated). The occurrence of IgG anti-lactoferrin was also confirmed by western blotting, revealing a single reaction band with an antigen of molecular weight about 80 000 and corresponding to rabbit anti-human leukocyte lactoferrin (Fig 2).

One patient originally included in the study had acute colitis and a high level of IgG anti-lactoferrin at the first sampling occasion, and a further raised anti-lactoferrin level in a later serum sample (not illustrated). This patient was, however, excluded from the study since her symptoms were explained by infection with *Campylobacter jejuni*.

Figure 3 illustrates the lack of correlation between levels of IgG anti-human lactoferrin and IgG anti-bovine lactoferrin as tested by ELISA with 10 sera positive in the anti-human lactoferrin and 12 sera negative in the same test.

Figure 4 shows the occurrence of IgA antibodies directed against α antigen, myeloperoxidase, and human lactoferrin.

Raised levels of IgA anti- α antigen were seen in 12 of 51 (24%) sera from Crohn's disease ($p < 0.001$), in 5 of 11 (45%) sera from patients with primary sclerosing cholangitis ($p < 0.001$), but in only 2 of 21 (10%) sera from patients with ulcerative colitis (NS). The levels of IgA anti-myeloperoxidase did not differ from the control group in any of the disease states, whereas IgA anti-lactoferrin was significantly ($p < 0.05$) more common in Crohn's disease, although it was seen only in a minority of the patient sera (5 of 51 = 10%). IgA anti-lactoferrin was found in significantly ($p < 0.001$) increased frequency also in ulcerative colitis (7 of 21 = 33%), but not in sclerosing cholangitis (1 of 11 = 9%).

The levels of IgA-ANCAs were not explained by the occurrence of agglutinating rheumatoid factors (not illustrated).

Figure 5 shows the frequency of positive IgG and/or IgA tests for either anti- α antigen, or anti-myeloperoxidase, or anti-lactoferrin in sera from blood donors, Crohn's disease, ulcerative colitis, and primary sclerosing cholangitis. Taken together, positive IgG tests for one or more of the three granulocyte antigens were found in 11% (24 of 218) control sera, in 19% (10 of 52) of Crohn's disease (NS), in 67% (16 of 24) of ulcerative colitis ($p < 0.001$), and in 67% (8 of 12) of primary sclerosing cholangitis sera; IgA anti-granulocyte antibodies were found in 7% (15 of 211) of the controls, in 29% (15 of 51) of Crohn's

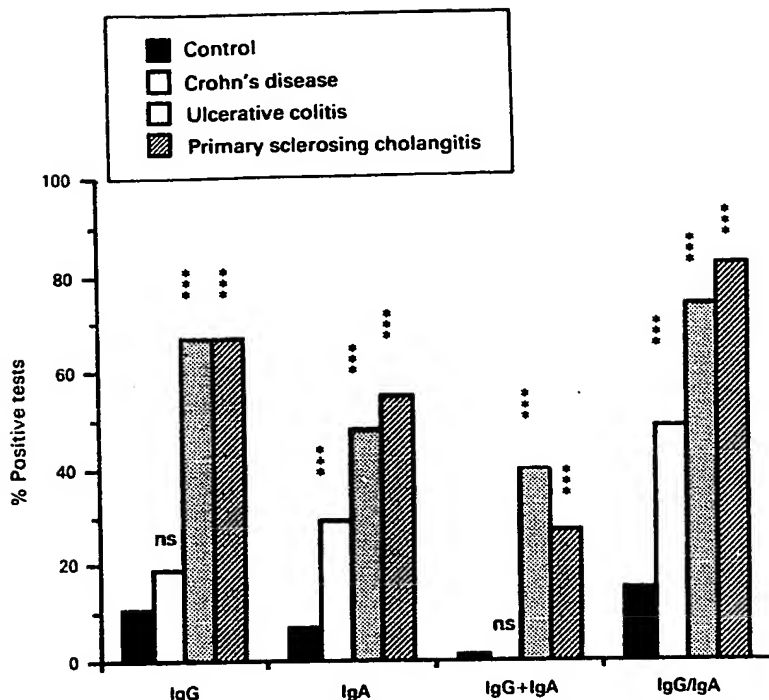


Figure 5: Diagram summarising the ELISA tests for IgG and/or IgA autoantibodies against all granulocyte antigens tested – that is, α antigen, myeloperoxidase, and human lactoferrin.

disease patients ($p < 0.001$), in 48% (10 of 21) of ulcerative colitis patients ($p < 0.001$), and in 55% (6 of 11) of primary sclerosing cholangitis subjects ($p < 0.001$). Antibodies of either IgG or IgA class were found in 49% of the sera from patients with Crohn's disease ($p < 0.001$), in 74% of the sera from patients with ulcerative colitis ($p < 0.001$), and in 82% of the cholangitis sera ($p < 0.001$).

There was no obvious correlation between disease activity or extraintestinal disease (arthritis, arthralgia, skin involvement) and the presence of IgG or IgA anti-granulocyte

antibodies. The levels of IgG and IgA anti-granulocyte antibodies did not correlate to the total serum levels of IgG and IgA (not illustrated).

Figure 6 shows the GS-ANA/P-ANCA immunofluorescence pattern of cytocentrifuged ethanol-fixed human polymorphonuclear neutrophil granulocytes after staining with rabbit anti-human milk lactoferrin. This, taken together with the western blot analysis (Fig 2), shows that antibodies against human milk lactoferrin recognise human leukocyte lactoferrin and vice versa.

Discussion

In this study we report high frequencies of IgG anti-lactoferrin antibodies (corresponding to P-ANCA) in sera from patients with ulcerative colitis and primary sclerosing cholangitis. In Crohn's disease, however, serum anti-lactoferrin antibodies of IgG were rarely detected, and then only in patients with colonic disease manifestations. IgA anti-lactoferrin antibodies were, however, found in some cases of both ulcerative colitis and Crohn's disease.

Several different types of autoantibodies have been described in inflammatory bowel disease and primary sclerosing cholangitis.^{11, 13, 19, 21, 22} Although none of the autoantibodies have been proved to be of pathogenetic significance, it has been shown, both in Crohn's disease and in ulcerative colitis, that IgG and complement can be present on the apical surface of enterocytes *in vivo*,^{23, 24} possibly indicating a pathogenetic role for anti-epithelial/anti-brush border antibodies in inflammatory bowel disease. In this connection, and considering the high frequency of anti-lactoferrin antibodies found in the present study, it is interesting to note that lactoferrin has been reported to bind to intestinal brush border via a specific receptor.²⁵ Antibodies of IgA class directed against *Saccharomyces cerevisiae* are common in Crohn's disease but not in ulcerative colitis, and determination of such antibodies may be of diagnostic help.¹¹ It is possible that many of the anti-microbial antibodies described in inflammatory bowel disease and other inflammatory disease states, may in fact be reflections of immunisation against microbial heat-shock proteins, which has been implied in the pathogenesis of several autoimmune disease states²⁶ and which may induce anti-lactoferrin antibodies²⁷ (see below).

Wakefield *et al* presented evidence that vasculitis and microthrombosis in mesenteric vessels can be important pathogenetic factors in inflammatory bowel disease.^{28, 29} Furthermore, P-ANCA/GS-ANA, which occurs in several primary vasculitis diseases, can frequently be demonstrated in inflammatory bowel disease and primary sclerosing cholangitis also.^{11, 13} P-ANCA/GS-ANA is more common in ulcerative colitis than in Crohn's disease,^{11, 13, 30} which is confirmed in the present study by the high frequency of IgG anti-lactoferrin antibodies in ulcerative colitis and primary sclerosing cholangitis but not in Crohn's disease. In Crohn's disease the frequency of IgG anti- α antigen and anti-myeloperoxidase antibodies did not differ

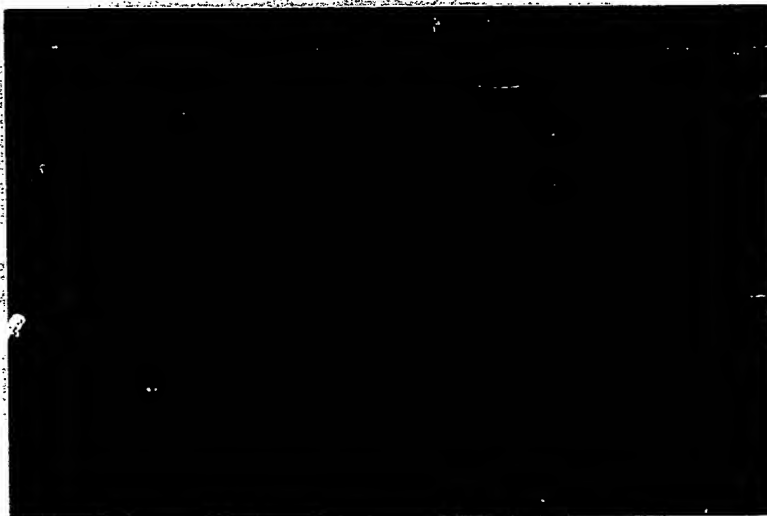


Figure 6: Granulocyte specific antinuclear antibodies/perinuclear staining anti-neutrophil cytoplasm antibodies (GS-ANA/P-ANCA) IIF staining pattern achieved after incubation of anti-human milk lactoferrin antibodies with cytocentrifuged, ethanol fixed human polymorphonuclear granulocytes.

statistically from the control group, although in a few instances the antibody levels were surprisingly high. In both ulcerative colitis and primary sclerosing cholangitis the frequency of positive IgG anti- α /anti-myeloperoxidase tests differed statistically from the control group, but in general the levels were low and, considering the small number of patients with positive anti- α /anti-myeloperoxidase tests, the relevance of these results is uncertain. However, it is interesting that these types of ANCAs were found at all, since they are considered to be reliable markers of primary systemic vasculitis.⁷ ANCAs of IgA class have been reported in Henoch-Schönleins purpura and IgA nephropathy, although these findings are sometimes explained by the presence of rheumatoid factor.¹¹ The appearance of antibodies directed against α antigen in some cases of ulcerative colitis (IgG) and in Crohn's disease (IgA) could possibly favour the hypothesis of primary vasculitis as a pathogenetic factor in these diseases.¹⁶⁻¹⁸

Lactoferrin is an iron binding protein that occurs abundantly not only in the specific granules of granulocytes,⁷ but also in tears, milk, and secretions at mucosal surfaces.¹⁴⁻¹⁶ Raised levels of circulating lactoferrin are seen during active inflammatory disease.¹⁷ It exerts antibacterial effects by depriving bacteria of iron required for growth, and it has anti-inflammatory properties - for example, by preventing complement activation through inhibition of classical C3 convertase.¹⁴ Lactoferrin can also prevent the formation of hydroxyl radicals by iron binding.¹⁴⁻¹⁶ Lactoferrin may thus be of great importance as a non-specific anti-phlogistic defence factor at the primary immunological barriers. It has been shown experimentally that binding of lactoferrin by anti-lactoferrin antibodies increases the amount and duration of hydroxyl radical formation by granulocytes.¹⁹ Hypothetically, anti-lactoferrin autoantibodies could, by counteracting the anti-inflammatory effects of lactoferrin, aggravate and prolong mucosal inflammation induced by several different mechanisms, and the antibodies may therefore have pathogenetic significance even though their occurrence does not seem to correlate with disease activity. Anti-lactoferrin antibodies may also have pathogenetic effects by activation of primed granulocytes infiltrating the gut mucosa or adhering to vessel walls, in analogy with the effects of other types of ANCA.^{5,40,41} In addition, mucosal lactoferrin/anti-lactoferrin complexes may stimulate intestinal goblet cells to excessive mucus secretion, as do other immune complexes.⁴² On the other hand, the occurrence of anti-lactoferrin autoantibodies may, of course merely be an epiphenomenon without pathogenetic significance. The fact that the individual types of ANCA were found only in a minority ($\leq 50\%$) of the sera tested is an argument in favour of this. On the other hand, consumption of circulating antibodies cannot be ruled out in the seronegative instances.

The origin of anti-lactoferrin autoantibodies is unknown. Although bovine and human lactoferrin have molecular and antigenic similarities,⁴³ immunisation of experimental animals with bovine lactoferrin seldom seems to result in

cross immunisation against human lactoferrin,⁴⁴ and the lack of correlation between levels of anti-human lactoferrin and anti-bovine lactoferrin shown in this study contradicts the hypothesis that cross immunisation against dietary bovine lactoferrin explains the appearance of anti-human lactoferrin autoantibodies. An exciting possibility is the antigenic mimicry between the 65 kD mycobacterial heat-shock protein (hsp-65) and human lactoferrin, and the fact that immunisation against hsp-65 results in the production of anti-lactoferrin antibodies.⁴⁵ Mycobacterial infection and immunisation against mycobacterial antigens/hsp-65 have been implicated in several autoimmune disease states, both experimental and clinical.^{30,31,41-45} Considering the occurrence of IgA anti-lactoferrin antibodies in some cases of Crohn's disease in the present study, we find it interesting that IgA (but not IgG) anti-hsp-65 antibodies have been reported in Crohn's disease.⁴⁶ Furthermore, the registration of anti-lactoferrin antibodies in a case of *Campylobacter* colitis in this study is interesting with regard to the possibility of cross immunisation against microbial antigen(s). We therefore intend to analyse additional sera from patients with infectious gastroenteritis for the presence of anti-lactoferrin antibodies.

In conclusion, we have shown high frequencies of IgG anti-lactoferrin antibodies in ulcerative colitis, and primary sclerosing cholangitis, but not in Crohn's disease, whereas IgA autoantibodies directed against lactoferrin were found in some cases of both ulcerative colitis and Crohn's disease. We suggest that anti-lactoferrin may be of pathogenetic significance by counteracting the antiphlogistic properties of lactoferrin at mucosal surfaces, thereby aggravating and/or sustaining mucosal inflammation initiated by other factors.

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Non-invasive investigation of inflammatory bowel disease

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Abstract

The assessment of inflammatory activity in intestinal disease in man can be done using a variety of different techniques. These range from the use of non-invasive acute phase inflammatory markers measured in plasma such as C reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) (both of which give an indirect assessment of disease activity) to the direct assessment of disease activity by intestinal biopsy performed during endoscopy in association with endoscopic scoring systems. Both radiology and endoscopy are conventional for the diagnosis of inflammatory bowel disease (IBD). However these techniques have severe limitations when it comes to assessing functional components of the disease such as activity and prognosis. Here we briefly review the value of two emerging intestinal function tests. Intestinal permeability, although ideally suited for diagnostic screening for small bowel Crohn's disease, appears to give reliable predictive data for imminent relapse of small bowel Crohn's disease and it can be used to assess responses to treatment. More significantly it is now clear that single stool assay of neutrophil specific proteins (calprotectin, lactoferrin) give the same quantitative data on intestinal inflammation as the 4-day faecal excretion of ¹¹¹Indium labelled white cells. Faecal calprotectin is shown to be increased in over 95% of patients with IBD and correlates with clinical disease activity. It reliably differentiates between patients with IBD and irritable bowel syndrome. More importantly, at a given faecal calprotectin concentration in patients with quiescent IBD, the test has a specificity and sensitivity in excess of 85% in predicting clinical relapse of disease. This suggests that relapse of IBD is closely related to the degree of intestinal inflammation and suggests that targeted treatment at an asymptomatic stage of the disease may be indicated.

Subject headings inflammatory bowel diseases; permeability; NCAM; membrane glycoproteins

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INTRODUCTION

Distinguishing irritable bowel syndrome from inflammatory bowel disease

Gastroenterologists are often faced with the diagnostic

difficulty of differentiating patients with the irritable bowel syndrome (IBS) from those with organic intestinal pathology, in particular inflammatory bowel disease (IBD). Many symptoms are common to both conditions including abdominal pain, bloating, excessive flatus and altered bowel habit while other clinical features such as a predominance of diarrhoea and rectal bleeding will increase the likelihood of organic disease. Although symptoms are a surprisingly good guide to a diagnosis, most clinicians proceed to and rely on laboratory tests to aid in the differential diagnosis. Certainly, fulfilling the ROME criteria^[1,2] and having a normal full blood count, routine biochemical screening, ESR and CRP are reassuring indicators pointing to IBS. As a result a number of investigators^[1,3,4] have recommended a straightforward approach to evaluation and treatment of patients with IBS based on the use of the Rome criteria as a means of cost effective management. Despite this the use of the Rome criteria has not been universal and is largely confined to use as entry criteria into research studies of patients with IBS. The concern for gastroenterologists is that some patients with organic intestinal disease will be incorrectly diagnosed if excess reliance is placed upon these criteria. They may therefore feel compelled to exclude all organic disease using invasive diagnostic investigations as objective evidence for there being no other significant pathology. This has significant implications for health care costs as well as exposing patients to the inherent risks associated with invasive procedures.

Managing inflammatory bowel disease

Once IBD is diagnosed the treatment involves induction and subsequently maintenance of remission based largely on clinical disease activity indices^[5,6] and the physicians global assessment of well-being. The problem with the use of clinical disease activity scores is that they are a composite of quantitative subjective symptoms that are affected by non inflammatory processes such as fibrous strictures, fistulas and previous surgical intervention. As a guide to clinical decision making, many clinicians therefore use nonspecific laboratory tests to document relapse of disease and radiology and radio isotopic techniques to distinguish between actively inflamed disease and fibrotic strictures. In addition a number of blood tests (erythrocyte sedimentation rate (ESR), orosomucoid, C-reactive protein (CRP), platelet, and white cell counts, IL-6, TNF- α , IL-1 β)^[7-11] which reflect the systemic consequences of inflammation, have been proposed as predictors and/or markers of clinical relapse of IBD with varying degrees of success. However, the overall predictive values of these different variables in identifying patients at risk of relapse have in general been disappointing. This is possibly due to the fact that these measures are non-specific, affected by a variety of non-intestinal diseases^[12] and most importantly do not measure the intestinal inflammation directly. Patients with clinically active IBD can have normal serological inflammatory indices while clinically quiescent disease may be associated with abnormal blood tests. In particular, there is a major discrepancy between severity of

symptoms and macroscopic evaluation of disease activity in patients with Crohn's disease limited to the colon.

Intestinal function tests

Although imperfect the above approach to diagnosis and management of patients with IBD remains the norm and in general it works well for the vast majority of patients. However, few would argue with the notion that there is scope for improvement. Where is such improvement to come from? Recently, investigators have turned to direct tests of intestinal function. Such tests provide new, direct and different information. They have the potential to be used as a diagnostic screen for intestinal disorders as well as providing prognostic information for the behaviour of the disease. At present there are three kinds of intestinal function tests that could fulfill the above promise, two of which (intestinal permeability and white cell scans) have a 20 year history. The third, namely direct assay of faeces for inflammatory markers, we suspect has the greatest potential. There follows a brief outline of how these tests can provide information that is not obtainable by other methods and their possible use in the day-to-day management of patients with IBD.

INTESTINAL PERMEABILITY

Permeability refers to that property of a membrane that enables passage of a solute by unmediated diffusion. The diffusion of a solute across a simple membrane is determined by the structure of the membrane (in terms of its composition, charge, thickness, etc.), the physicochemical properties of the solute (like molecular size, shape, charge and solubility) and its interaction with the media or solvent. Intestinal permeability is assessed non-invasively *in vivo* by measuring urinary excretion of orally administered substances. The ideal permeability probe is water-soluble, non-toxic, non-degradable and not metabolised before, during or after permeating the intestine^[13]. The probes should preferably not be naturally present in urine, be completely excreted in the urine following intravenous administration and be easily and accurately measurable. Fordtran *et al*^[14] were instrumental in the development of ideas for assessing intestinal permeability in man but it was Menzies who introduced oligosaccharides as test substances for the non-invasive assessment of intestinal permeability^[15] in 1974, and later formulated the principle of differential urinary excretion of orally administered test substances^[16]. The importance of the differential urinary excretion principle is that it overcomes most if not all the problems associated with the use of a single test substance, where urinary excretion is dependent on a number of pre- and post-mucosal factors as well as intestinal permeability. The differential principle advocates that a nonhydrolyzed disaccharide (i.e. lactulose) and a monosaccharide (L-rhamnose or mannitol) are ingested together. As the pre- and post-mucosal determinants of their excretion affects the two test substances equally and the differential 5 hour urinary excretion ratio (ratio of lactulose/L-rhamnose) is not affected by these variables the urinary excretion ratio becomes a specific measure of intestinal permeability.

Tests of intestinal permeability were initially designed to allow reliable non-invasive detection of patients with untreated coeliac disease^[16]. The tests have since come to be viewed as synonymous with assessing intestinal barrier function. In clinically active small bowel Crohn's disease the vast majority of patients (>95%) have an increase in the differential urinary excretion of ingested di-/monosaccharides (lactulose/L-rhamnose or mannitol) and half of those with Crohn's colitis are abnormal^[17]. These figures are

marginally improved with the use of ⁵¹CrEDTA, which requires a 24-hour, as opposed to a 5-hour urinary collection. The vast majority of patients with ulcerative colitis have normal small intestinal permeability when assessed by these methods. However, tests of intestinal permeability have not found widespread application as screening tests to discriminate between patients with Crohn's disease and IBS. The reason for this is probably that the urinary sugar analysis is time consuming and demanding, and there may be some concern that the tests lack specificity being abnormal in a variety of small intestinal diseases (Table 1). At first sight the test appears to identify a number of "clinically irrelevant" diseases, which usually translates into disease for which no treatment is available, but in practice the tests seem often to identify small intestinal pathology where none was previously expected, thus expanding the number of identifiable small bowel pathologies.

There have been attempts to use intestinal permeability as an index of disease activity in Crohn's disease. In general these have been disappointing because the degree of increase in the differential urinary excretion of lactulose/L-rhamnose or the excretion of ⁵¹CrEDTA is dependent on localisation and extent of disease within the small bowel as well as activity of the inflammation^[18]. Abnormalities in intestinal permeability may, however, be used as a predictor of imminent relapse of quiescent Crohn's disease. Three studies have now shown that, in patients with Crohn's disease in clinical remission, an increased intestinal permeability can predict those at significant risk of relapse of disease in the next few months^[17-19]. The strength of this association is difficult to assess from the published studies. Nevertheless, less than 20% of those with normal intestinal permeability appear to relapse over the ensuing 6 months. Interestingly, elevated levels of IL-6 in serum, which can be viewed as a surrogate marker of intestinal inflammation, also has a predictive value for relapse of Crohn's disease^[20], but receiver operating curve (ROC) analysis shows relatively low sensitivity and specificity (70 and 50%, respectively). The permeability ratio differs from such indices in that it is not based on concentrations of plasma proteins but rather represents functional changes in the intestinal mucosa, a direct consequence of intestinal inflammation.

The clinical implications of these findings are discussed later.

WHITE CELL SCANS AND FAECAL EXCRETION

Intense neutrophil recruitment to the intestinal mucosa is a feature common to inflammatory bowel diseases^[20]. When a patient's own radiolabelled neutrophils are re-injected they migrate to sites of acute inflammation as well as to the liver, spleen and bone marrow^[21]. Segal, Savarymuttu and Chadwick were instrumental in the introduction, validation and application of the ¹¹¹Indium white cell technique for use in gastroenterology^[22,23]. The technique visualises inflamed segments of bowel and quantitates the degree of inflammatory activity^[23,24].

A number of studies have established that abdominal scans are abnormal in virtually all patients with active IBD; their accuracy in localisation of disease and distinguishing between actively inflamed and fibrous stricturing disease has implications for treatment. It was suggested that the technique could be used to discriminate, with an accuracy approaching 100%, between patients with IBD and IBS at the first outpatient visit. In practice this suggestion was not followed up with relevant research.

When combined with measurement of the 4 day faecal

excretion of labelled white cells for quantitation of the inflammatory activity the technique becomes a formidable tool for research and investigation. The faecal excretion of the labelled white cells quantitate inflammation accurately and can be used to document therapeutic efficacy of various treatments in IBD^[25,27]. It has also been used to define a number of enteropathies (NSAIDs, alcohol, chronic renal failure, hypogammaglobulinaemia, HIV-AIDS, etc.) where none were suspected or impossible to demonstrate by techniques other than perhaps the intestinal permeability tests (Table 1)^[28]. The method is not disease specific, resembling that of the permeability tests, but it is specific for intestinal inflammation. This is not a drawback as it is a simple matter to distinguish between the inflammatory activity in patients with IBD and the above enteropathies, colonic cancer, diverticulitis, etc., since patients with active IBD have excretion values often an order of magnitude higher than the others.

Table 1 Some conditions reported to be associated with increased intestinal permeability

Nonsteroidal anti-inflammatory drugs	Inflammatory bowel disease
Alcohol	Ankylosing spondylitis
Renal failure	Celiac disease
Abdominal radiation	Intestinal ischaemia
Cytotoxic drug treatment	Hypogammaglobulinaemia
Abdominal surgery	HIV infection
Fasting	Endotoxaemia
Total parenteral nutrition	Multorgan failure
Food allergy	Diabetic diarrhoea
Multiple sclerosis	Scleroderma
Cystic fibrosis	Reactive arthritis
Recurrent abdominal pain of childhood	Intestinal infections/ bacterial overgrowth
Neomycin	Whipple's disease
Acute and chronic liver disease	Sarcoidosis

Why has the white cell technique not been universally adapted for use as a diagnostic screen in IBD, and to assess disease activity? It requires expensive labeling facilities including labelling cabinets. The labeling procedure is time consuming, taking over 2 hours. The cost of isotope and material is in excess of £200 (US \$300) and the radiation dose is not trivial if abdominal scans are carried out, being equivalent to that of a barium enema^[29,30]. A complete 4-day faecal collection is also demanding and unpleasant for patients, occasionally requiring hospital admission.

Other methods have attempted to build on this success. One such is ^{99m}Tc labeling of white cells^[31]. This is purported to give superior quality abdominal scintigraphy (which is not clinically important), but does not allow late (>4 hours) scanning, because the label comes off and is excreted into the bowel independent of white cell excretion. Furthermore a faecal collection provides no quantitative information on intestinal inflammation (as the Technetium comes off the white cells and is excreted in faeces) and the labeling requires the same facilities as the white cells.

Newer techniques include E-selectin scanning^[32]. This method is derived from the more conventional labelled white cell scintigraphy, but uses a labelled antibody to E-selectin, which is over-expressed in endothelial cells at sites of inflammation. It has the advantage of studying a more fixed entity that (unlike white cells) will not be shed at a variable rate into the bowel lumen and is applicable to the occasional patient with intestinal inflammation who is neutropenic.

In our opinion, the greatest impact that the white cell

technique has had is that it ① emphasised that if a sensitive method is to be established for assessing intestinal function there are no shortcuts. Neurologists assess spinal fluid, respiratory physicians assess sputum, urologists urine and the gastroenterologist needs to come terms with the fact that faecal analysis is essential to obtain maximal information about the state of the intestine. ② emphasised that there is life beyond morphological assessment of the gut (x-ray and colonoscopic studies). ③ raised the possibility of dramatically changing our views on the treatment of IBD. Many patients with IBD in full clinical remission are shown to have significant intestinal inflammation^[27,33]. At present treatment is non-specifically directed at maintaining remission (5-ASA, azathioprine, etc.). It seems highly probable that those patients with substantial inflammatory activity should be targeted for more aggressive therapy, in particular if they can be shown to be at significant risk of clinical relapse of disease. The analogy with the treatment of rheumatoid arthritis springs to mind. Here, first line treatment is directed to wards reducing the acute inflammatory component of the disease followed by a number of second line agents that can alter the natural history of the disease, reduce the frequency, duration and severity of relapses as well as reducing the joint damage.

FAECAL MARKERS

Faecal analysis is unpleasant but has been with us for a long time. Measure of electrolytes and osmolality helped in the differential diagnosis of diarrhoea in children. Faecal fats were a widespread screening test for steatorrhoea for a while and faecal occult bloods have become the yardstick for colorectal screening with which other methods need to be compared. An improvement on these techniques was the introduction of radioisotopically labelled compounds (labelled red blood cells, proteins, white cells) which provided quantitative and functional data and which was event specific (blood loss, inflammation, protein losing enteropathy, etc.) but non-specific for disease.

The inflamed hyperpermeable mucosa of patients with inflammatory bowel disease is associated with increased protein loss into the bowel lumen^[34]. Studies using radiolabelled proteins have demonstrated that there is faecal protein loss in patients with active Crohn's disease and it may therefore be a useful marker of disease activity. Other studies have shown faecal α 1 antitrypsin clearance to be a useful indicator of protein losing enteropathy^[35] and that in patients with inflammatory bowel disease, 72 hour faecal clearance of α 1 antitrypsin is a useful method for quantitating intestinal protein loss^[36,37]. Faecal clearance of α 1 antitrypsin correlates with that of ⁵¹Cr-albumin, and moderate rectal bleeding does not affect the α 1 antitrypsin determination^[36]. Random faecal α 1 antitrypsin levels have been shown to be as useful as more prolonged collection in measuring Crohn's disease activity^[38] and correlated with several other laboratory measures that have been proposed as indicators of Crohn's disease activity^[39].

Concerns about costs, radiation, and the need for prolonged faecal collections all worked against these techniques for routine use, although many remain very important for research studies. The idea then emerged that it might be possible to assay for cell proteins or substances that are specifically associated with a certain cell type and which would then provide information on a specific component of the inflammatory cascade. Ferguson's Edinburgh group was instrumental in expanding this idea^[40]. Concerned about bacterial degradation of markers they used a whole gut lavage method involving ingestion of polyethylene-based purgatives

(Kleenprep or GoLately) for obtaining clear liquid faecal samples for analysis. The analysis took to various markers, such as immunoglobulins, neutrophils-specific elastase, and haemoglobin. Separate studies showed that Crohn's disease could be identified with ease, and that the method had a greater sensitivity for colorectal cancer than the conventional faecal occult blood technique. Ideally suited for research, the method has as yet not found wide application for routine screening purposes, possibly because of the drawback of patients needing to ingest large volumes of liquid.

Direct analysis of markers in faeces would be a major advance on this method. Here the problem is initially the bacterial degradation of the marker necessitating swift sample handling. One such marker, TNF, has been successfully used in children and in HIV infection in adults^[41,42]. However, it is now clear that it is not necessary for the marker to be completely non-degraded, provided that the antibody (most of these assays are ELISA's or radio immunoassay) is directed at an epitope of the molecule which resists degradation. One such assay is that for lactoferrin^[43]. Lactoferrin is a relatively specific marker for neutrophils, in which it is present in cytoplasmic granules.

Faecal calprotectin

The greatest experience with analysis of faecal proteins is with calprotectin^[44,45]. It accounts for up to 50% of the neutrophilic cytosolic protein while being resistant to colonic bacterial degradation. It is easily measured in faeces by a commercially available ELISA.

Calprotectin was first isolated from granulocytes by Fagerhol *et al*^[46] and named L1 protein, but was later named calprotectin upon identification of its calcium binding and antimicrobial properties^[50]. The protein is a heterocomplex protein consisting of two heavy (L1H) chains and one light (L1L) chain^[51] which are non-covalently linked^[52]. Calprotectin appears to play a regulatory role in the inflammatory process^[53] and functions in both an antimicrobial^[50,54] and antiproliferative capacity^[55-57]. It has both bactericidal and fungicidal properties with minimal inhibitory concentrations comparable to those of many antibiotics^[50]. It is released from the cells during cell activation or cell death. The C-terminal sequence of the L1H chain has been shown to be identical to the N-terminus of peptides known as neutrophil immobilising factors (NIF)^[58]. It has been suggested that NIF activity of the L1H chain depends upon its phosphorylation^[59] and that such an activity of calprotectin could be important for the accumulation of granulocytes, while calprotectin released from dead neutrophils, macrophages and epithelial cells might exert antimicrobial activity, possibly by depriving microorganisms of zinc^[60,61]. Calprotectin may inhibit metalloproteinases^[62] which may also involve the deprivation of zinc suggesting that it may limit their participation as enzymatic cofactors for invading organisms. Interest in calprotectin as a marker for inflammation in the gut followed the realisation that ¹¹¹Indium labelled granulocyte scans could be used to both visualise and quantitate the acute inflammation in the gut of patients with inflammatory bowel disease^[20,23]. These findings led to the idea that an increased influx of granulocytes into the intestinal mucosa in conditions of inflammation might give increased levels of proteins from such cells in faeces.

Others^[63] have demonstrated that eosinophilic granulocytes are the main cellular source of calprotectin in the normal gut mucosa. However, relatively high levels of calprotectin are found in the stools of normal individuals-

about six times the plasma levels (which are about 0.5mg/L). This is compatible with data suggesting that in normal individuals most circulating neutrophils migrate through the mucosal membrane of the gut wall and thereby terminate their circulating life^[64]. Subsequent lysis within the gut lumen and release of cytosolic calprotectin thereby accounts for the median faecal levels of 2.0mg/L seen in healthy controls^[44,65]. The diagnostic use of faecal calprotectin in a broad spectrum of intestinal diseases has been studied by a number of groups with remarkable agreement between the results to date.

Inflammatory bowel disease

It is almost possible to extrapolate all the findings obtained with the white cell faecal excretion technique to the calprotectin method. Both techniques correlate with histopathological assessment of disease activity in ulcerative colitis and there is a very good correlation between the 4-day faecal excretion of white cells and faecal calprotectin concentrations^[33,45], a correlation which is maintained when single stool calprotectin concentrations are used as opposed to 1 or 4 day collections. The faecal calprotectin concentration has a narrow normal range with an upper limit of 10mg/L. As with the white cells, faecal calprotectin has potential as a screening procedure to differentiate between patients with IBD and IBS and it may be useful for documenting a fall in intestinal inflammation in response to successful treatment of disease. Calprotectin concentration is rarely within the normal range in patients with IBD despite full clinical remission and is therefore a highly sensitive method for detecting such patients irrespective of disease activity. In over 100 patients with Crohn's disease of varying severity and activity only 4 had normal calprotectin concentrations^[33].

Since the method is so much simpler than the white cell technique, requiring only a single stool sample, extraction and an ELISA, it has potential as a screening test to distinguish between patients with IBD and IBS in an outpatient setting. One study in over 225 patients showed that a cut off of 30mg/L had a 100% sensitivity and 94% specificity for this purpose^[33]. Another showed that this was also the case when over 600 unselected consecutive patients were studied. Indeed a patient presenting with positive ROME criteria and a normal faecal calprotectin has virtually no chance of having IBD^[66]. As a result of these studies it is now our practice not to investigate such patients by radiology or colonoscopy with considerable cost saving implications. The white radiolabelled cell technique demonstrated reduced intestinal inflammation in response to 5-ASA treatment and elemental diets. We have shown (unpublished) that improvement in calprotectin parallels the improvement in the excretion of labelled white cells in response to treatment with elemental diets. These techniques prove to be much more reliable and reproducible than the changes in clinical disease indices. It seems likely that the assay of faecal calprotectin will become an integral part of the assessment of therapeutic efficacy of the acute inflammation in future treatment trials in patients with IBD.

Apart from screening and assessing response to treatment, the faecal calprotectin has a further major advantage over the white cell labeling technique in predicting relapse of IBD. It has been shown that, in patients with clinically quiescent IBD (ulcerative colitis and Crohn's disease), faecal calprotectin values above 50mg/L may be used to predict clinical relapse of disease within a few months with over 80% sensitivity^[67]. Symptoms of inflammatory bowel disease often appear to be the direct consequence of the inflammatory process itself and often vary dependent upon

the location of the inflammation. Most patients with quiescent IBD have low-grade inflammation^[7] and it is possible that symptomatic relapse occurs only when the inflammatory process reaches a critical intensity. Furthermore, as inflammation is a continuous process it may be that direct assessment of the level of inflammatory activity may provide a quantitative pre-symptomatic measure of imminent clinical relapse of the disease.

The clinical implications of this, if substantiated, are considerable as it might offer targeted treatment at an earlier stage, with less side effects, to avert the relapse, as well as assessment of new therapeutic strategies to maintain symptomatic remission^[60]. At present this is done with some degree of success with the rather indiscriminate use of sulphasalazine, 5-ASA and azathioprine, all of which are associated with side effects. However the calprotectin method offers guidance as to whom to treat at this stage and with what kind of vigour. Theoretically such treatment should lead to a dramatic reduction in the frequency and severity of clinical relapses with an improvement in the patient's quality of life.

In addition, the identification of patients at high risk of relapse will improve the design of clinical trials to assess the efficacy of therapeutic regimes designed to maintain patients in remission. In most such trials, patients studied tend to be a heterogeneous mix of those with high and low risk of relapse. This introduces possible bias when assessing the response to a particular treatment regime due to the imbalance of high risk patients in each treatment arm. Stratification by risk group using faecal calprotectin would reduce the possibility of such a bias. It is also possible that a lack of power in detecting a response to treatment may be due to the study of a large number of patients at low/intermediate risk of relapse, in whom all treatments may show the same efficacy, and therefore clinical trials studying a homogenous high risk group may be more powerful in detecting a difference in treatment efficacy.

Much work remains to be done, some is already on its way, but what is clear is that gastroenterologists need to move with the times and start thinking along the lines that rheumatologists do, that is, to implement treatments that alter the natural history of the disease. We are now in possession of tests that have the potential to revolutionise our approach to treatment of patients with IBD. There are some hurdles to overcome. The most frequent criticism of the "faecal" tests is that they are unacceptable to patients and unpleasant to work with.

The faecal calprotectin and lactoferrin methods are the first wave of techniques that allow non-invasive assessment of specific and selective cellular components of the intestinal inflammatory cascade. At present these are useful for a variety of purposes, outlined above, but it is likely that it will be possible to estimate the participation of other cells. Many other cells of the inflammatory cascade are numerically increased in biopsy specimens from patients with a variety of gastroenterological conditions. Some, such as mast cells and eosinophils, are thought to play a central role in mediating intestinal allergic reactions^[61]. However, both types of cell are found to be activated in a number of other gastrointestinal inflammatory diseases such as inflammatory bowel disease, coeliac disease, eosinophilic gastroenteritis^[62] and collagenous colitis^[70], suggesting that both cell types may be involved in the pathogenesis of chronic intestinal inflammation. It may therefore be possible, as for neutrophils and calprotectin, to identify mast cell granule proteins, such as tryptase and chymase, in faecal samples and use them as markers of a specific component of the intestinal inflammatory response.

The long-term objective might be to fully automate a faecal sample assay method that provides specific information on the activity of acute inflammation (neutrophils), chronic inflammation (T-cells) and allergy (mast cells).

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Both celiac disease and inflammatory bowel disease (IBD) are characterized by chronic diarrhea and the presence of distinct (auto)antibodies. In the present study we wanted to determine the prevalence of serological markers for inflammatory bowel disease, i.e., perinuclear antineutrophil cytoplasmic antibodies (pANCA) and/or anti-Saccharomyces cerevisiae antibodies (ASCA), in 37 patients with biopsy-confirmed celiac disease (Marsh IIIb/c). The majority of the patients was positive for IgA (auto)antibodies typically associated with celiac disease, i.e., antiendomysium antibodies (EMA) (86.5%), antigliadin antibodies (AGA) (73%), and antirecombinant human tissue transglutaminase antibodies (rh-tTGA) (86.5%). Four patients with selective IgA deficiency could be identified by analyzing EMA, AGA, and rh-tTGA for the IgG isotype. The prevalence of pANCA and ASCA, markers that are used for IBD, was unexpectedly high in our cohort of patients with celiac disease: 8 patients were positive for pANCA (IgG) and 16 patients were positive for ASCA (IgG and/or IgA). These results indicate that the presence of pANCA or ASCA in the serum of patients with chronic diarrhea does not exclude celiac disease. A prospective study is required to determine whether pANCA and/or ASCA identify patients at risk for developing secondary autoimmune disease.

☐ 1: Am J Gastroenterol. 1999 Dec;94(12):3513-26.

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Fecal eosinophil granule-derived proteins reflect disease activity in inflammatory bowel disease.

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OBJECTIVES: The aims of this study were: 1) to examine whether the fecal levels of eosinophil granule-derived proteins reflect disease activity in inflammatory bowel disease (IBD); and 2) to examine the extracellular release of these proteins from eosinophils and their stability in feces by an in vitro study. **METHODS:** We investigated 42 patients with ulcerative colitis (UC), 37 patients with Crohn's disease (CD), and 29 control subjects. The stool samples were collected at 4 degrees C over 48 h and were homogenized. The fecal levels of eosinophil cationic protein (ECP) and eosinophil protein X (EPX) were measured by radioimmunoassay. Fecal Hb (Hb), alpha1-antitrypsin (AT), and lactoferrin (Lf) were also measured by ELISA. **RESULTS:** Fecal ECP and EPX concentrations were significantly increased in both active UC and active CD compared to inactive UC and inactive CD, respectively. Fecal EPX concentration correlated with the fecal Hb, AT, and Lf concentrations more closely than fecal ECP concentration. Even in the inactive stage, CD patients who relapsed within the following 3 months showed higher fecal ECP and EPX concentrations compared to the patients who did not. EPX was released extracellularly more efficiently than ECP (18.6% vs 6.3%, after incubation for 15 min at 25 degrees C). EPX was more stable in the feces than ECP. **CONCLUSIONS:** The measurement of eosinophil granule-derived proteins in feces is useful for evaluating disease activity and predicting relapse in patients with IBD. EPX may be more suitable than ECP as a fecal eosinophil marker.

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Fecal Eosinophil Granule-Derived Proteins Reflect Disease Activity in Inflammatory Bowel Disease

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RESULTS: Fecal ECP and EPX concentrations were significantly increased in both active UC and active CD compared to inactive UC and inactive CD, respectively. Fecal EPX concentration correlated with the fecal Hb, AT, and Lf concentrations more closely than fecal ECP concentration. Even in the inactive stage, CD patients who relapsed within the following 3 months showed higher fecal ECP and EPX concentrations compared to the patients who did not. EPX was released extracellularly more efficiently than ECP (18.6% vs 6.3%, after incubation for 15 min at 25°C). EPX was more stable in the feces than ECP.

CONCLUSIONS: The measurement of eosinophil granule-derived proteins in feces is useful for evaluating disease activity and predicting relapse in patients with IBD. EPX may be more suitable than ECP as a fecal eosinophil marker. (Am J Gastroenterol 1999;94:3513–3520. © 1999 by Am. Coll. of Gastroenterology)

INTRODUCTION

Eosinophils are involved in a broad range of diseases such as allergic, inflammatory, and malignant disorders (1, 2). The specific granules of the eosinophils contain a number of highly cationic proteins such as eosinophil cationic protein (ECP), eosinophil protein X (EPX)/eosinophil-derived neurotoxin (EDN), major basic protein (MBP), and eosinophil

peroxidase (EPO). These proteins have potent cytotoxic action and are released from the cells after activation and stimulation of the cells (3). Intestinal mucosa of the patients with inflammatory bowel disease (IBD) is characterized by epithelial cell damage and infiltration of various inflammatory cells. The inflammatory cells include neutrophils, lymphocytes, plasma cells, macrophages, and eosinophils. Neutrophils contain various proteins such as lactoferrin, PMN (PMN)-elastase, myeloperoxidase, and lysozyme in their granules. We previously reported that the fecal levels of these neutrophil-derived proteins increased in feces in patients with active IBD and reflect disease activity (4, 5). We then considered that the measurement of fecal eosinophil-derived proteins may provide information regarding eosinophil involvement in the pathological process of IBD. The aims of the present study were: 1) to examine whether the fecal levels of eosinophil granule-derived proteins (ECP and EPX) reflect disease activity and predict relapse in patients with IBD; 2) to compare fecal eosinophil granule-derived proteins and other fecal markers of disease activity; and 3) to examine the extracellular release of eosinophil granule-derived proteins from eosinophils and their stability in feces by an *in vitro* study.

MATERIALS AND METHODS

Extracellular Release of ECP and EPX by Eosinophils In Vitro

A quantity of 500 μ l of heparinized blood samples from four healthy subjects were incubated at 25°C for 15 min. Plasma was obtained by centrifugation at 2000 g for 5 min. The concentration of ECP and EPX in plasma was measured by a radioimmunoassay (RIA) kit (Pharmacia and Upjohn, Kalamazoo, MI). To determine the total amount of ECP and EPX in whole blood, Triton X-100 (1% final concentration) was added to the heparinized blood samples, and the concentrations of ECP and EPX were measured. The percentage of ECP and EPX released extracellularly was obtained by the following equation: Percent extracellular release (%) = $(a/b) \times 100$, where a is the concentration (ng/ml) in plasma after incubation and b is the concentration (ng/ml) in whole blood treated with Triton X-100.

Subjects

A total of 42 patients with UC (age 34.5 ± 15.5 yr [mean \pm SD]; eight with proctitis, 11 with left-sided colitis, 23 with pancolitis) and 37 patients with CD (age 29.1 ± 13.0 yr, 13 with the small intestine type, 18 with the small and large intestine type, six with the large intestine type) were evaluated. Crohn's colitis and UC were differentiated endoscopically and histologically. UC was defined as being in the active phase if the patients showed clinical symptoms (rectal bleeding, diarrhea) and/or an inflamed colonic mucosa (Baron's grade 2 or 3) at colonoscopy (6). Disease activity in CD was assessed according to the Crohn's disease activity index (CDAI), in which a score of >150 was considered to represent active disease (7). Regarding medication, sulfasalazine or 5-aminosalicylate was administered in 41 of 51 active UC samples, in 33 of 38 inactive UC samples, in 38 of 50 active CD samples, and in 34 of 49 inactive CD samples. Prednisolone was administered in 19 of 51 active UC samples, in 19 of 38 inactive UC samples, in 14 of 50 active CD samples, and in 15 of 49 inactive CD samples. The control group consisted of 29 subjects (age 40.6 ± 21.5 yr) with no endoscopic abnormality in the upper or lower digestive tract.

Informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

Method of Stool Collection and

Measurement of Fecal ECP, EPX, Hb (Hb), $\alpha 1$ -Antitrypsin ($\alpha 1$ -AT), and Lactoferrin (Lf)

Patients were instructed to defecate directly into a polystyrene container (diameter 15 cm, depth 12 cm). The stool samples, stored at 4°C over 48–72 h, were homogenized with a small amount of water, and then stored at -80°C until the time of measurement. The fecal levels of ECP and EPX were measured by an RIA kit (Pharmacia and Upjohn). An RIA kit for ECP was a kind gift from Pharmacia and Upjohn. The optimal sample dilution for assay was first examined. Samples diluted at 1:40 or more with PBS showed good linearity of the assay system, whereas samples diluted at 1:20 with PBS showed a higher level than theoretically expected. This was considered to be due to possible interference present in the stool. Therefore, the samples were diluted at 1:40 or more with PBS. Coefficient of variations in intraday assay and interday assay for ECP and EPX were $<15\%$. Fecal Hb, $\alpha 1$ -AT and Lf were measured by ELISA as described previously (5, 8). Fecal Hb and $\alpha 1$ -AT are useful markers of disease activity in UC and CD, respectively (5). Fecal Lf is useful particularly for evaluating the presence of minimal intestinal inflammation (8).

Stability of ECP and EPX in Feces

To examine the stability of these proteins in the feces, homogenized stool samples were stored at 4°C , 25°C , and 37°C for 0, 12, 24, and 48 h before freezing and subsequent analysis.

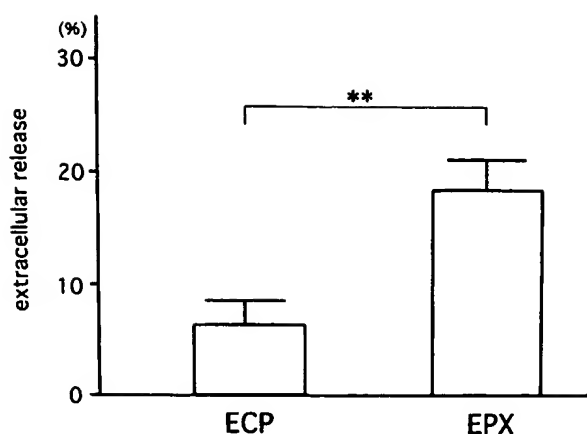


Figure 1. Extracellular release of ECP and EPX by eosinophils *in vitro*. Student's *t* test was used for statistical analysis: ** $p < 0.01$.

Contribution of Fecal Concentrations of ECP and EPX in the Inactive Phase of the Disease in Predicting Subsequent Relapse

Among the patients described in the Subjects section, 30 patients with UC (age 33.4 ± 15.0 yr [mean \pm SD]; six with proctitis, nine with left-sided colitis, and 15 with pancolitis) and 35 patients with CD (age 29.1 ± 13.5 yr, 13 with the small intestine type, 17 with the small and large intestine type, and five with the large intestine type) were used as subjects to examine the contribution of fecal concentrations of ECP and EPX in predicting subsequent relapse. At the time of stool collection, all these patients had been at an inactive stage for >2 months. The patients were divided into "relapse patients" and "nonrelapse patients." There were no significant intergroup differences in the distributions of age and type of disease. Relapse was considered to occur when the disease became active. The definitions of active disease were mentioned above. A patient who relapsed within the 3 months after collecting stool samples was defined to be a "relapse patient." A patient who did not relapse within the 3 months after collecting stool was defined as a "nonrelapse patient." Fecal concentrations of ECP, EPX, Hb, $\alpha 1$ -AT, and Lf were compared between "relapse patients" and "nonrelapse patients."

Statistical Analysis

Values were expressed as means \pm SE. Student's *t* test was used for statistical analyses. Linear regression analysis was used for correlation analysis. All *p* values were two-tailed; values of $p < 0.05$ were considered statistically significant.

RESULTS

Extracellular Release of ECP and EPX by Eosinophils In Vitro

As shown in Figure 1, the extracellular release of ECP and EPX were $6.3 \pm 2.0\%$ and $18.6 \pm 2.4\%$, respectively. The extracellular release of EPX was more efficient than that of

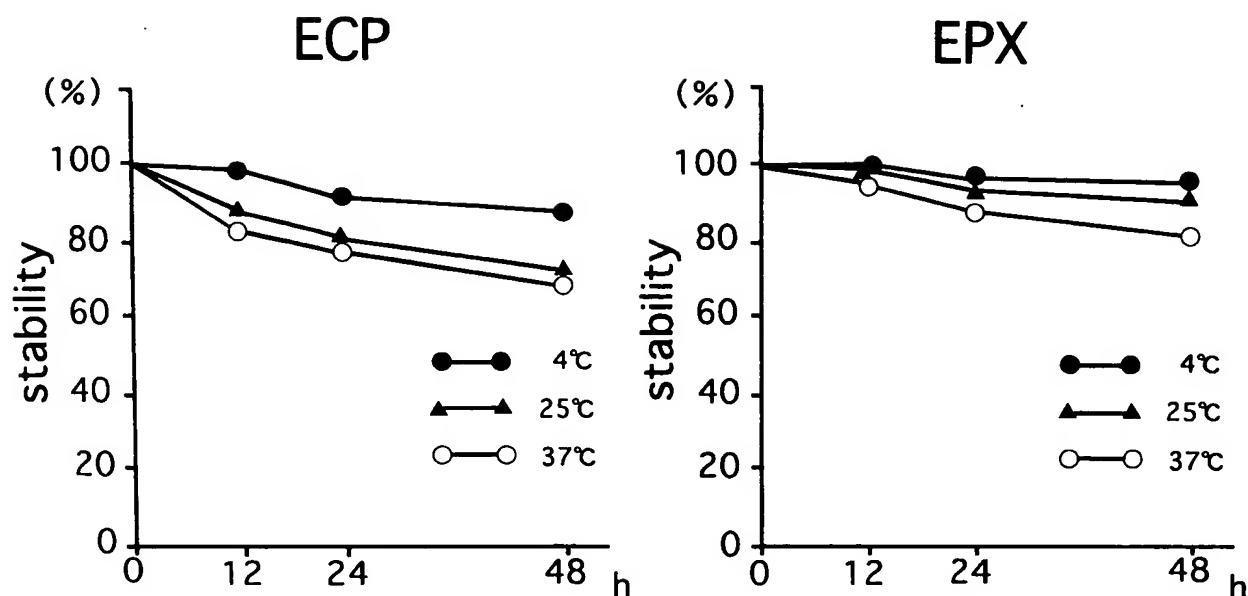


Figure 2. Stability of fecal ECP and EPX in feces: filled circles, at 4°C; &filled triangles, at 25°C; open circles, at 37°C.

ECP. After treatment with Triton X-100, the mean concentrations of ECP and EPX in whole blood were 138 and 171 $\mu\text{g/ml}$, respectively.

Stability of Fecal ECP and EPX

The data are shown in Figure 2. The concentration of ECP and EPX was expressed as a percentage of the original

concentration at 0 h. EPX was more stable in the feces than was ECP (92.2% vs 73.0% at 25°C for 48 h).

Levels of Fecal ECP and EPX in Patients With UC and CD

Fecal concentrations of ECP and EPX in patients with UC and CD were demonstrated in Figures 3 and 4. In patients

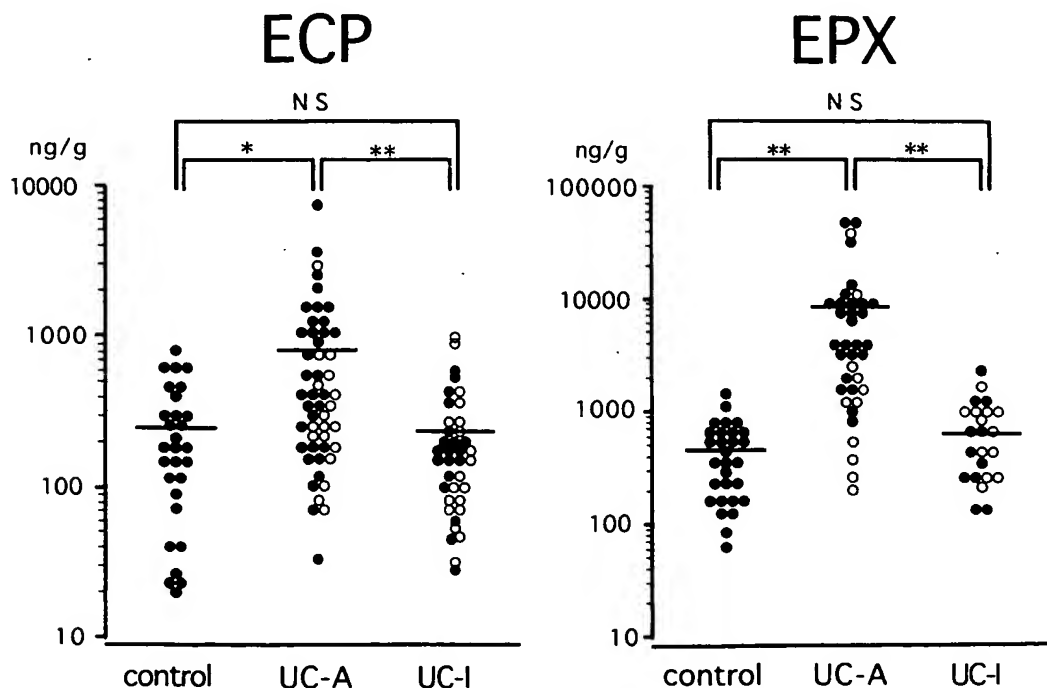


Figure 3. Fecal concentrations of ECP and EPX in patients with UC. UC-A, ulcerative colitis (active phase); UC-I, ulcerative colitis (inactive phase). Patients who did not receive corticosteroids are indicated by filled circles, and patients who received corticosteroids are indicated by open circles. When the patients who received corticosteroids were excluded from the subjects, the same differences were found. Student's *t* test was used for statistical analyses: * $p < 0.05$; ** $p < 0.01$. NS = not significant.

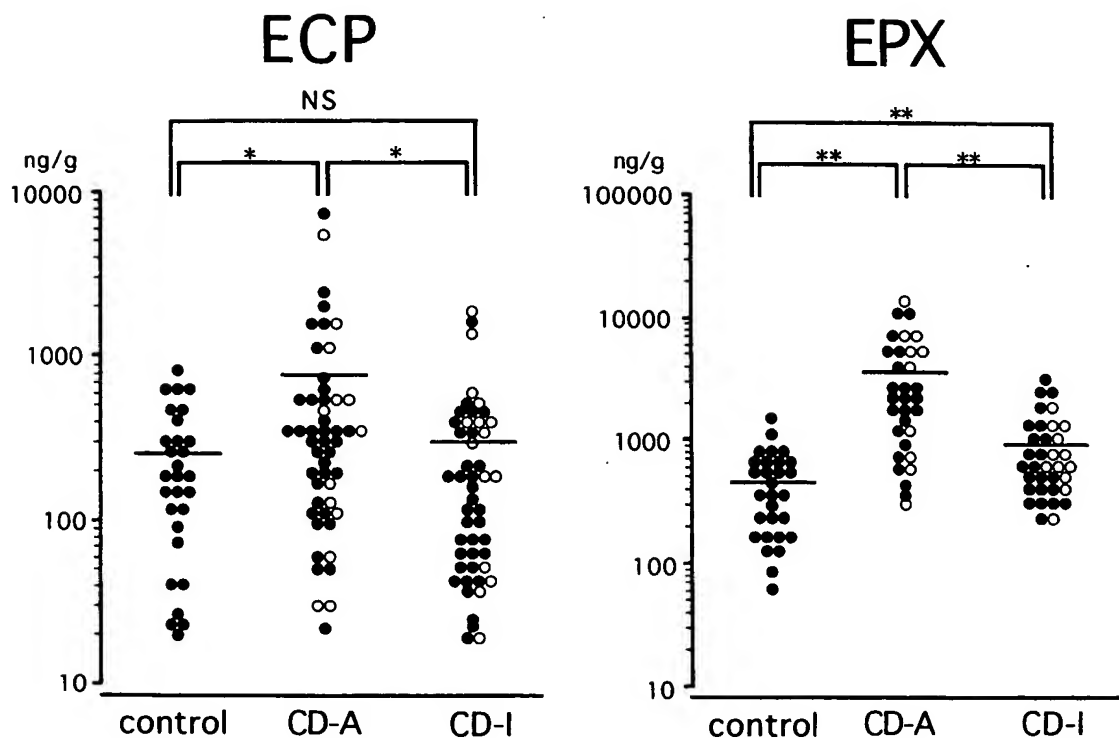


Figure 4. Fecal concentrations of ECP and EPX in patients with CD. CD-A; Crohn's disease (active phase); CD-I, Crohn's disease (inactive phase). Patients who did not receive corticosteroids are indicated by filled circles, and patients who received corticosteroids are indicated by open circles. When the patients who received corticosteroids were excluded from the subjects, the same differences were found. Student's *t* test was used for statistical analyses: * $p < 0.05$; ** $p < 0.01$. NS = not significant.

with active UC, inactive UC, active CD, inactive CD and in the control subjects, fecal ECP concentrations were 822.1 ± 169.3 , 239.3 ± 34.9 , 785.4 ± 194.9 , 301.9 ± 52.4 , and 248.4 ± 39.3 , respectively. Respective fecal EPX concentrations were 8576.3 ± 2043.5 , 654.4 ± 118.4 , 3522.9 ± 604.7 , 910.2 ± 123.7 , and 455.0 ± 61.4 . In both UC and CD, there were significant differences between the active and inactive phases, and between the active phases and the control. When patients who received corticosteroids were excluded from the subjects, the same differences were found. Fecal EPX showed more evident difference between the disease group and the control than fecal ECP. Patients with active UC were further divided into two groups: patients with massive bleeding (daily fecal Hb excretion > 0.5 g/day) and patients without massive bleeding. Patients with active CD were divided into two groups according to their CDAI score. In patients with active UC (massive bleeding, $n = 10$), active UC (no massive bleeding, $n = 41$), active CD (CDAI > 200 , $n = 12$) and active CD ($150 < \text{CDAI} \leq 200$, $n = 37$), the fecal ECP concentrations were 945.4 ± 236.8 , 792.0 ± 203.4 , 884.0 ± 578.1 , and 682.2 ± 176.4 , respectively. The respective fecal EPX concentrations were 22308.7 ± 6735.0 , 4788.0 ± 1162.5 , 4098.4 ± 1191.5 , and 3240.1 ± 742.5 . There were significant correlations between the fecal levels of eosinophil granule-derived proteins and daily fecal Hb excretion in UC ($r = 0.400$, $p < 0.001$ for ECP, $r = 0.745$, $p < 0.001$ for EPX). Between the active

UC with massive bleeding and those without massive bleeding, there was a significant intergroup difference in the fecal EPX concentration, but not in the fecal ECP concentration. In CD, there was a significant correlation between the fecal EPX concentration and CDAI ($r = 0.505$, $p < 0.001$), but not between the fecal ECP concentration and CDAI ($r = 0.202$, $p = 0.053$). Between active CD (CDAI > 200) and active CD ($150 < \text{CDAI} \leq 200$), however, there was no significant intergroup difference in fecal ECP or EPX concentrations.

Relationship Between Fecal ECP and EPX in Patients With UC and CD

The values are shown in Figure 5. There were significant correlations on the logarithmic scale between ECP and EPX concentrations in UC and CD. UC showed better correlation than CD. Most of the samples that showed a normal ECP concentration and a high EPX concentration were obtained from active patients (18 of 21 in UC, and 16 of 25 in CD).

Relationship Between Fecal Eosinophil Markers and Hb or $\alpha 1$ -AT in Patients With UC and CD

We previously found that fecal Hb and $\alpha 1$ -AT were useful markers of disease activity in UC and CD, respectively. We therefore examined the relationship between concentrations of the eosinophil markers (ECP, EPX) and the concentrations of Hb or $\alpha 1$ -AT. As shown in Figure 6, there were

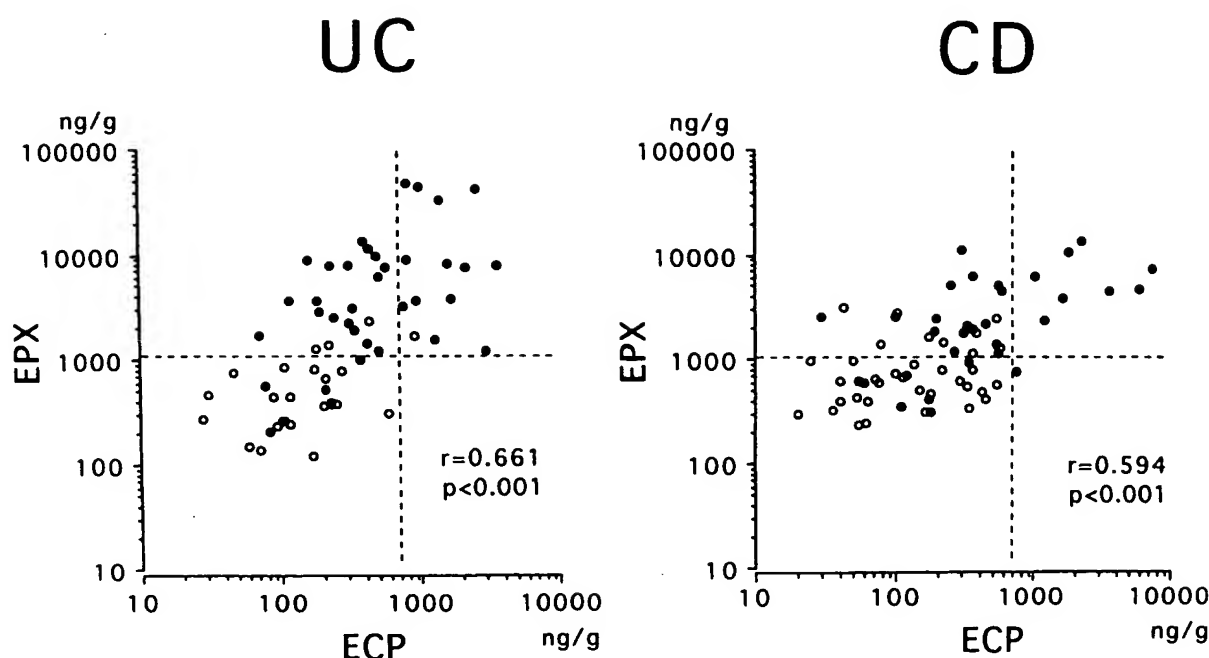


Figure 5. Relationship between fecal ECP and EPX in patients with UC and CD. Dotted lines, mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g for ECP, EPX, respectively). Open circles, inactive phase; filled circles, active phase.

significant correlations on the logarithmic scale between eosinophil markers and Hb or α 1-AT in UC and CD. Fecal EPX concentrations correlated with the fecal Hb or AT concentrations more closely than did the fecal ECP concentrations. Good correlations were obtained in UC, but not in CD.

Relationship Between Fecal Eosinophil Markers and Lf in Patients With UC and CD

The values are shown in Figure 7. There were significant correlations on the logarithmic scale between fecal eosinophil markers and Lf in UC and CD. UC showed better correlation than CD. Fecal EPX concentrations correlated with the fecal Lf concentrations more closely than did the fecal ECP concentrations.

Predictive Value of Fecal Eosinophil Markers for Subsequent Relapse

As shown in Figure 8, in patients with CD, fecal ECP and EPX showed significant differences between "relapse patients" and "nonrelapse patients." In UC, there were no significant differences between "relapse patients" and "nonrelapse patients." However, UC patients with high levels of fecal eosinophil markers relapsed. In contrast to fecal eosinophil markers, fecal Hb, α 1-AT, and Lf did not show significant differences between "relapse patients" and "nonrelapse patients" in either UC or CD (data not shown).

DISCUSSION

In patients with various intestinal diseases including IBD, increased eosinophil counts and enhanced eosinophil acti-

vation were found in the intestinal mucosa (9–11). Because there has been no appropriate method to assess the eosinophil activation of the intestinal mucosa, the role of eosinophils in the pathogenesis of IBD remains unclear. Routine histological observation of the intestinal mucosa misses degranulated eosinophils and is not sufficient for the assessment of eosinophil activation. Immunohistochemistry using antibody against eosinophil granular proteins is useful for assessing eosinophils of the intestinal mucosa (9). However, colonoscopy is required to obtain biopsies of the intestinal mucosa. In contrast, fecal tests are safe and can be performed repeatedly. To establish the measurement of fecal eosinophil markers is important from a clinical point of view. Eosinophils produce and release various inflammatory mediators. Among them, a number of highly cationic proteins present in the granules of the eosinophils are specific for eosinophils. In the present study, therefore, we focused on fecal eosinophil granule-derived proteins as a marker of eosinophil activation of intestinal mucosa.

There have been two reports concerning fecal eosinophil granule-derived proteins (13, 14). Comparing the two studies, Berstad *et al.* reported approximately 20-fold higher fecal levels of ECP in feces than did Bischoff *et al.* The present study demonstrated that the assay system had good linearity and a reasonable coefficient of variations. The fecal ECP levels of the present study were closer to that reported by Berstad *et al.* Both reports showed high fecal levels in patients with active IBD. However, they did not characterize inactive patients who had high fecal levels of eosinophil markers. The usefulness of fecal eosinophil markers for predicting relapse was not examined. In addition, we com-

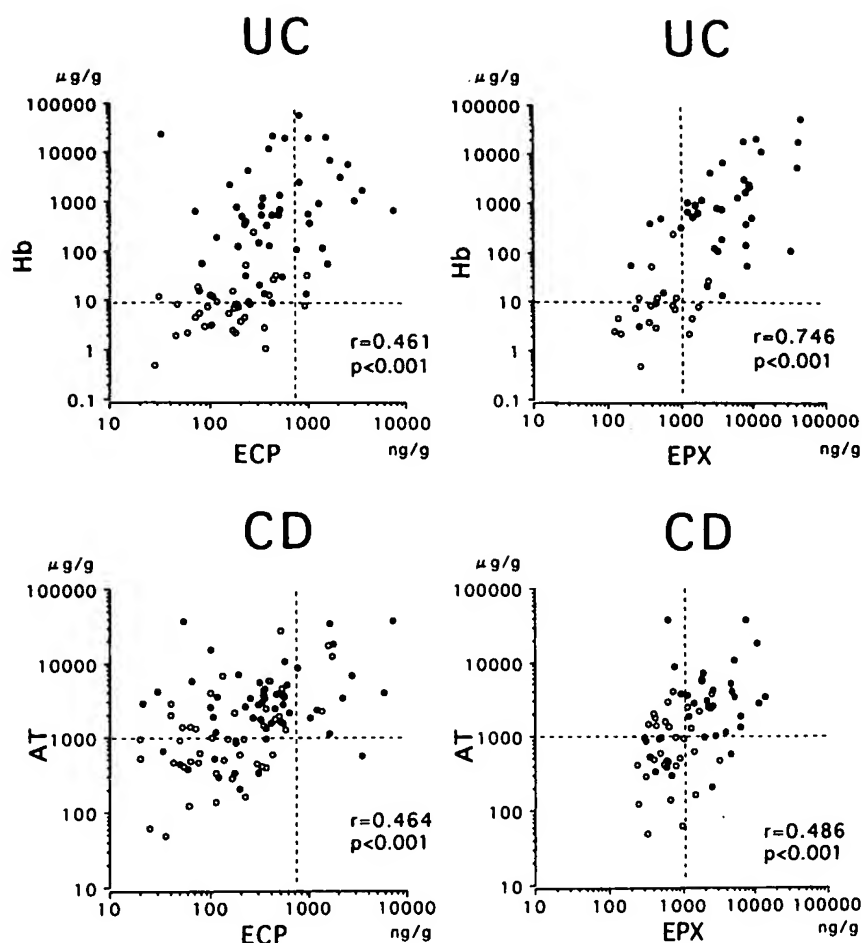


Figure 6. Relationship between fecal eosinophil markers and Hb or $\alpha 1$ -AT in patients with UC and CD. Dotted lines, mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g, 9.7 μ g/g, and 995.5 μ g/g for ECP, EPX, Hb, and $\alpha 1$ -AT, respectively). Open circles, inactive phase; filled circles, active phase.

pared ECP and EPX in terms of their stability in feces as well as their extracellular release.

It is well known that eosinophil granular proteins are released extracellularly when the cells are stimulated. To constitute a superior fecal marker of intestinal eosinophil activation, a protein should be released efficiently from the cells, as well as being stable in feces. From this point of view, EPX seemed to be a more suitable marker than ECP, because it was released from the cells more efficiently and was more stable in the feces.

Fecal Hb, $\alpha 1$ -AT, and neutrophil-derived proteins are markers for disease activity in IBD (4, 5, 8). Fecal Hb is good for UC, whereas $\alpha 1$ -AT is good for CD. Lf is a useful marker for evaluating the presence of minimal intestinal inflammation of UC and CD. Hb and Lf were elevated in almost all of the patients with active UC, indicating that bleeding and mucosal neutrophil infiltration are common features of all patients with UC. In the present study, however, fecal EPX and ECP were not elevated in all of the patients with active UC, indicating that activation of intestinal eosinophils is not a common feature of all patients with

IBD. Fecal ECP and EPX levels reflect disease activity to some extent. However, fecal ECP and EPX levels may provide information on eosinophil activation of the intestinal mucosa, rather than on quantitative disease activity. Clinical features may be different between IBD patients who have activated intestinal eosinophils and IBD patients who do not. It would be interesting to note whether or not there are similarities in the clinical features of IBD patients who have activated intestinal eosinophils and patients with eosinophilic gastroenteritis. Furthermore, modified or optional medical treatment may be useful in the subgroup of IBD patients with strong activation of intestinal eosinophils. Drugs that inhibit the migration and activation of eosinophils may be useful as maintenance therapy to prevent recurrence in these patients (16, 17). Medical treatment may be a relevant factor affecting fecal ECP and EPX levels. In the present study, even if the patients who received corticosteroids are excluded from the subjects, fecal ECP and EPX levels were significantly increased in both active UC and active CD compared to inactive UC and inactive CD, respectively.

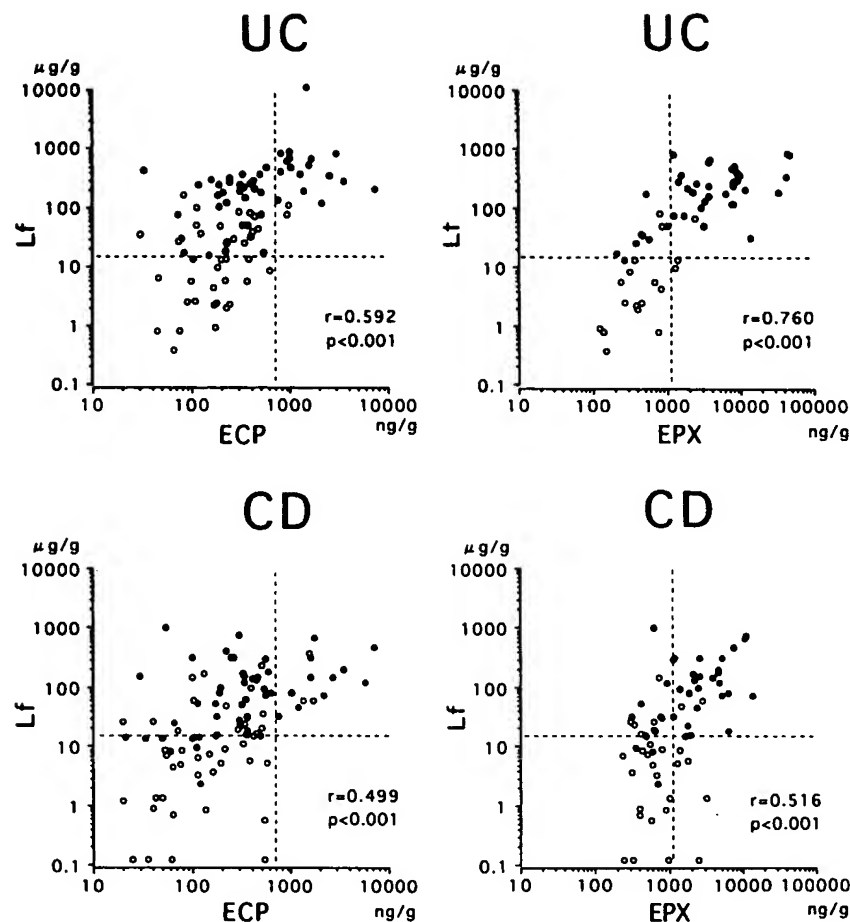


Figure 7. Relationship between fecal eosinophil markers and Lf in patients with UC and CD. Dotted lines, mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g, and 16.5 μg/g for ECP, EPX, and Lf, respectively). Open circles, inactive phase; filled circles, active phase.

UC and CD are relapsing and remitting diseases. Factors predisposing to recurrence are poorly understood. In the active phase of IBD, the participation of eosinophils in the pathophysiology is clear (17–20). It was reported that large numbers of eosinophils in the rectal mucosa during the active disease predict a benign course. However, it had been unknown whether eosinophils contribute to early mucosal damage in patients with IBD. Recently, Dubucquoi *et al.* showed that eosinophil infiltration was detected in early endoscopic recurrence cases after radical resection for CD (21). More recently, D'Haens *et al.* demonstrated that contact with intestinal fluids induced focal infiltration of mononuclear cells and eosinophils in the ileum of patients with CD (22). Our findings showed that patients with CD who relapsed clinically within the following 3 months showed higher fecal ECP and EPX concentrations even in the inactive phase. These findings indicate that eosinophil activation of the intestinal mucosa may trigger a flare-up of inflammation, which leads to clinical relapse. To address the accurate causal relation between eosinophil activation and relapse, the fecal levels of eosinophil markers should be examined serially and chronologically in patients with IBD.

It has been suggested that the results of immunological tests on extracts of feces do not represent the status of the gut humoral system (23). In the present study, there was substantial overlap of the fecal concentrations of EPX and ECP between the active and inactive phases. Analyses of whole gut lavage may be more accurate than fecal tests for estimating the total amount of mediators released into the gut lumen (14, 23), but the methods are complicated.

In conclusion, the measurement of eosinophil granule-derived proteins in feces is useful for evaluating the disease activity and predicting relapse in patients with IBD. EPX may be more suitable than ECP as a fecal eosinophil marker.

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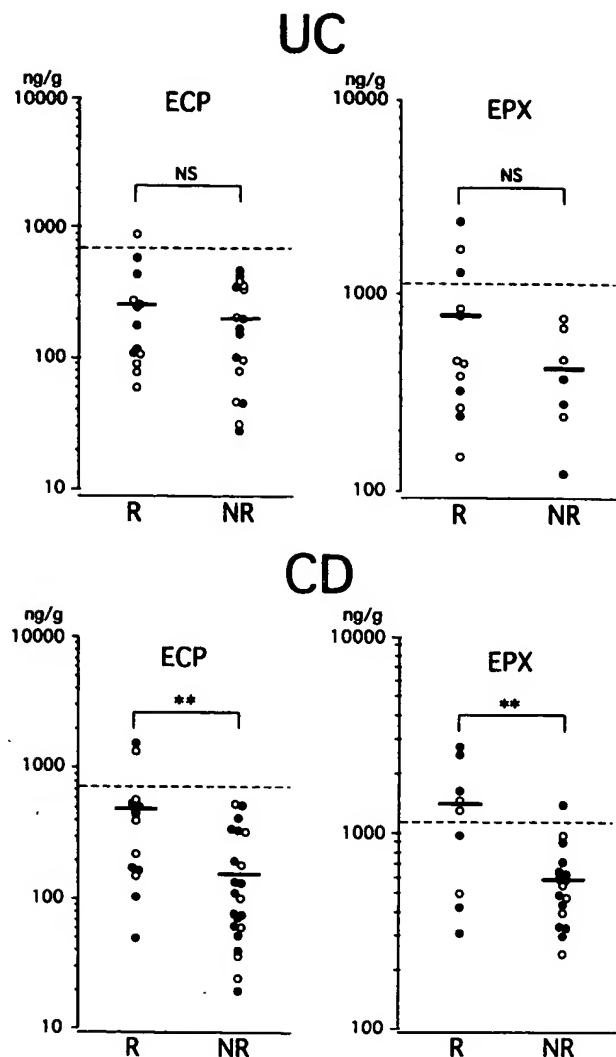


Figure 8. Fecal concentrations of ECP and EPX in inactive UC and CD: Comparison between relapse patients and nonrelapse patients. R = relapse within the following 3 months, NR = nonrelapse within the following 3 months. Dotted lines indicate mean + 2 SD of the control subjects (712.0 ng/g, 1115.8 ng/g for ECP, EPX, respectively). Patients who did not receive corticosteroids are indicated by filled circles, and patients who received corticosteroids are indicated by open circles. When the patients who received corticosteroids were excluded from the subjects, the same differences were found. ** $p < 0.01$. NS = not significant.

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Lactoferrin in Whole Gut Lavage Fluid as a Marker for Disease Activity in Inflammatory Bowel Disease: Comparison With Other Neutrophil-Derived Proteins

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OBJECTIVES: We investigated which neutrophil-derived proteins in whole gut lavage fluid (WGLF) most accurately reflect disease activity in inflammatory bowel disease.

METHODS: WGLF was obtained from patients undergoing whole gut lavage as a bowel preparation for colonoscopy. Twenty-seven patients with ulcerative colitis (UC), 23 patients with Crohn's disease (CD), and 35 control subjects were examined. The concentrations of lactoferrin, polymorphonuclear neutrophil elastase (PMN-E), myeloperoxidase, and lysozyme in WGLF were measured by ELISA. For the assessment of stability, WGLF samples were stored at 37°C for various periods.

RESULTS: In UC, the concentrations of lactoferrin, myeloperoxidase, and lysozyme in WGLF had good correlations with colonoscopic grading. Zero, 12, five, and 10 of 28 samples from active UC patients showed normal concentrations of lactoferrin, PMN-E, myeloperoxidase, and lysozyme, respectively. In CD, the concentrations of lactoferrin and myeloperoxidase had good correlations with the Crohn's disease activity index. Thirteen and seven of 36 samples from inactive CD patients (Crohn's disease activity index ≤ 150) showed high concentrations of lactoferrin and myeloperoxidase, respectively. Most of them (11/13, 6/7) were found to have ulceration by colonoscopy or small bowel x-ray. The ratio of the lactoferrin concentration in the WGLF supernatant to that in total WGLF was highest among these proteins in all disease groups and control subjects. Lactoferrin and myeloperoxidase showed good stability in WGLF, whereas PMN-E and lysozyme did not.

CONCLUSION: Lactoferrin is the most suitable of these proteins for use as a neutrophil-derived WGLF marker of intestinal inflammation. (*Am J Gastroenterol* 2002;97:360-369. © 2002 by Am. Coll. of Gastroenterology)

INTRODUCTION

It is very important to accurately evaluate intestinal mucosal inflammation in the management of patients with inflammatory bowel disease (IBD). Colonoscopy and biopsy are useful to assess the inflammation of the intestinal mucosa in patients with IBD, but these tests create a heavy burden for the patient and may exacerbate the mucosal inflammation (1, 2). In patients with Crohn's disease (CD), clinical activity indices such as the Crohn's disease activity index (CDAI) (3), the Simple Index (4), and the Dutch Activity Index (5) have been used. Although these indices remain useful for defining clinical remission/relapse, they do not always reflect intestinal mucosal inflammation.

Whole gut lavage is now widely used as a bowel preparation for colonoscopy or colorectal surgery (6, 7). The clear fluid passed after colonic cleaning is essentially a gut perfusate. Recently, the pathophysiologies of intestinal diseases have been investigated by measuring proteins in whole gut lavage fluid (WGLF) (8-13). It is well known that the measurements of fecal proteins are useful for investigating various pathophysiologies such as protein-losing enteropathy and mucosal inflammation. WGLF may have an advantage over feces as a sample for protein measurement for the following reasons:

1. WGLF contains smaller amounts of substances that interfere with assays.
2. Destruction of protein by digestive enzymes and bacterial proteases is less in WGLF because of its quick transit.
3. It is possible to estimate the rate of protein release from the mucosa, because the rate of fluid passage along the gut can be estimated.

Therefore, WGLF may reflect the intestinal mucosal inflammation in IBD more accurately than feces (14). Brydon and Ferguson (9) reported that measurement of Hb in WGLF not only detects intestinal bleeding, but also indicates intestinal

daily blood loss. Choudari *et al.* (10) showed that the concentration of IgG in WGLF correlated well with disease activity in patients with IBD.

It is important to evaluate intestinal bleeding and protein loss in patients with IBD. However, these conditions do not directly reflect intestinal mucosal inflammation (15–18). Neutrophil infiltration of intestinal mucosa could occur in a patient without bleeding. To assess intestinal mucosal inflammation in patients with IBD, therefore, neutrophil infiltration in intestinal mucosa and neutrophil transmigration into the gut lumen should be noted. Recently, it was demonstrated that sustained neutrophil transmigration induces apoptosis in intestinal epithelial cells (19). Histological examination of biopsy specimens is useful for the evaluation of neutrophil infiltration in intestinal mucosa. However, biopsy specimens are only parts of the gut. Furthermore, biopsy is not adequate for evaluating neutrophil transmigration, with the exception of crypt abscesses.

Neutrophil-granular proteins are released from neutrophils when the cells are activated. We therefore assumed that measurement of neutrophil-derived proteins in WGLF could reflect intestinal inflammation and be clinically applicable. There has been only one report concerning polymorphonuclear neutrophil elastase (PMN-E) in WGLF in patients with IBD (12). However, the correlation between PMN-E concentration and disease activity was not precisely investigated. Neutrophil-derived proteins other than PMN-E have not yet been investigated in WGLF. In the present study, the clear WGLF remaining in the rectum was collected at the beginning of colonoscopy and the concentrations of lactoferrin, PMN-E, myeloperoxidase, and lysozyme were measured by ELISA.

To investigate which of these four proteins most accurately reflects disease activity in IBD, we examined the relationship between these protein concentrations and the disease activity of the patient. The disease activity in UC was assessed according to the colonoscopic grading, and that in CD was assessed according to the CDAI. In patients with CD, we evaluated mucosal lesions via colonoscopy and small bowel x-ray. To investigate degranulation of neutrophils in the intestine, not only a WGLF supernatant (supernatant after centrifugation), but also unprocessed WGLF (total WGLF without centrifugation) were used as samples. Furthermore, the stability of these proteins in WGLF was examined with an *in vitro* study.

MATERIALS AND METHODS

Subjects

Twenty-seven patients with UC (age = 42.1 ± 12.3 yr [mean \pm SD]; five with proctitis, 14 with left-sided colitis, eight with pancolitis) and 23 patients with CD (age = 30.9 ± 10.1 yr; five with the small intestine type, 14 with the small and large intestine type, four with the large intestine type) were evaluated. Five of 27 UC patients and nine of 23

CD patients were hospitalized two or more times, and each admission was treated as an independent clinical course. The disease activity of UC was assessed according to the colonoscopic grading described below. This grading was based mostly on the sigmoidoscopic scale proposed by Matts (20):

- grade 1 = vascular pattern without contact bleeding
- grade 2 = loss of vascular pattern, mild granularity of the mucosa, mild contact bleeding, or mild erythema
- grade 3 = marked granularity, marked edema, marked erythema, mucopus, contact bleeding, or spontaneous bleeding
- grade 4 = ulceration of mucosa with hemorrhage

UC was defined as being in the active phase if the colonoscopic grade of the patient was 2, 3, or 4. Disease activity in CD was assessed according to the CDAI, in which a score of more than 150 was considered to represent the active phase. The control group consisted of 35 subjects (age = 45.2 ± 17.3 yr) with no demonstrated abnormality in the upper or lower digestive tract. Informed consent was obtained from each subject in accordance with the Helsinki Declaration.

Lavage Protocol

Patients drank a PEG-based lavage fluid (Niflec, Ajinomoto Pharma, Tokyo, Japan) at a rate of 250 ml every 15 min. This lavage fluid is isotonic and nonabsorbable. The composition of this fluid is the same as Golytely (Braintree Laboratories, Braintree, MA). Solid feces were passed, followed by liquid feces, and then several large volume discharges of virtually clear fluid. Clear fluid remaining in the rectum was obtained using an aspiration tube through the biopsy channel and then colonoscopy was performed. Some of the aliquots were centrifuged at 1000 g for 10 min at 4°C and the supernatants (WGLF supernatant) were stored at –80°C. The other aliquots were stored at –80°C, without centrifugation (total WGLF).

Measurements of Lactoferrin, PMN-E, Myeloperoxidase, and Lysozyme in WGLF by ELISA

Lactoferrin, PMN-E, myeloperoxidase, and lysozyme in WGLF were measured by ELISA as described previously (15, 17). Briefly, anti-human lactoferrin antibody (Dakopatts, Glostrup, Denmark), anti-human PMN-E antibody (Serotec, Oxford, United Kingdom), anti-human myeloperoxidase antibody (Dakopatts), or anti-human lysozyme antibody (Dakopatts) was placed into the wells of a 96-well microplate. The samples, diluted 10- to 1000-fold with 0.1 mol/L of Tris-HCl buffer (pH = 7.5) containing 0.1% bovine serum albumin and 0.2% sodium azide, were added to each well. After reaction at 37°C for 70 min, the wells were washed with water. The samples were then reacted with the respective ALP-labeled antibodies. After reaction at 37°C for 90 min the wells were washed with water. An enzyme reaction test was then performed, and color devel-

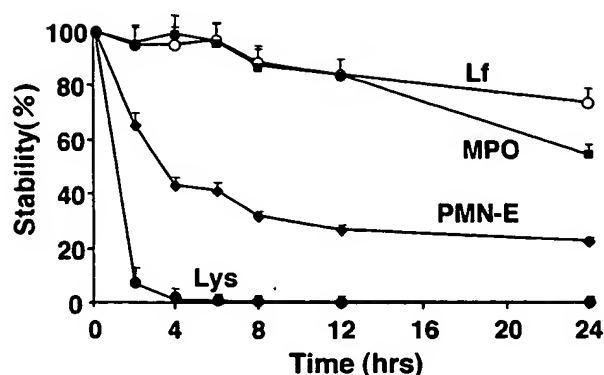


Figure 1. Stability of lactoferrin (○, Lf), PMN-E (◆), myeloperoxidase (■, MPO), and lysozyme (●, Lys) in WGLF at 37°C. Data are means \pm SEMs (n = 6).

opment was measured with a microplate colorimeter at 510/630 nm. Coefficients of variation in intra- and interday assays for these four proteins were less than 10% and 15%, respectively.

Stability of Lactoferrin, PMN-E, Myeloperoxidase, and Lysozyme in WGLF

To examine the stability of these proteins in WGLF, WGLF samples from active UC patients (n = 3) and active CD patients (n = 3) were stored at 37°C for 0, 2, 4, 6, 8, 12, and 24 h before freezing and subsequent analysis.

Statistical Analysis

Values are expressed as means \pm SEMs. The Student's *t* test was used for statistical analyses. Linear regression analysis was used for correlation analysis. All *ps* were two tailed; *p* values less than 0.05 were considered significant. Receiver operating characteristic (ROC) analysis was also used for the comparison of diagnostic efficiency with neutrophil-derived proteins in WGLF. The ROC curve is essentially a graph of the conditional probability of true positive responses by an observer in a detection experiment, *versus* the conditional probability of false positive response. Typically, the area under the ROC curve tends to be approximately 0.5 for an inappropriate technique and 1.0 for a perfect technique (21).

RESULTS

Stability of Lactoferrin, PMN-E, Myeloperoxidase, and Lysozyme in WGLF

The concentration of each neutrophil-derived protein in WGLF was expressed as a percentage of the original concentration at 0 h. The data are shown in Figure 1. Lactoferrin and myeloperoxidase had good stability in WGLF (more than 80% after 12 h), whereas PMN-E and lysozyme did not have good stability (PMN-E, less than 40% after 4 h; lysozyme, less than 10% after 2 h).

Concentrations of Neutrophil-Derived Proteins (Lactoferrin, PMN-E, Myeloperoxidase, and Lysozyme) in WGLF Supernatants

The WGLF supernatant contains neutrophil-derived proteins released from neutrophils. On the other hand, the pellet contains cells such as intestinal epithelial cells and neutrophils. Total WGLF is composed of a supernatant and pellet. The concentrations of lactoferrin, PMN-E, myeloperoxidase, and lysozyme in the WGLF supernatant are shown in Table 1 and Figure 2. In both UC and CD, the concentrations of these four neutrophil-derived proteins in the WGLF supernatant were significantly increased in the active phase relative to the inactive phase and control subjects.

Concentrations of Neutrophil-Derived Proteins (Lactoferrin, PMN-E, Myeloperoxidase, and Lysozyme) in Total WGLF

The concentrations of lactoferrin, PMN-E, myeloperoxidase, and lysozyme in total WGLF are shown in Table 2 and Figure 3. In both UC and CD, the concentrations of these four neutrophil-derived proteins in total WGLF were significantly increased in the active phase relative to the inactive phase and control subjects.

Relationship Between Protein Concentrations in WGLF and Colonoscopic Grading in UC

As shown in Figure 4, all four neutrophil-derived proteins in the WGLF supernatant had good correlations with colonoscopic grading. The correlation coefficients between colonoscopic grading and lactoferrin, PMN-E, myeloperoxidase, and lysozyme in WGLF supernatant were 0.884, 0.561, 0.883, and 0.76, respectively. In active UC (grade \geq 2), lactoferrin, PMN-E, myeloperoxidase, and lysozyme were

Table 1. Concentrations of Lactoferrin (Lf), PMN-E, Myeloperoxidase (MPO), and Lysozyme (Lys) in the WGLF Supernatant (Supernatant After Centrifugation) in UC and CD

	Control (n = 35)	UC-A (n = 28)	UC-I (n = 27)	CD-A (n = 23)	CD-I (n = 36)
Lf (μ g/ml)	0.54 \pm 0.09	16.92 \pm 4.34	0.86 \pm 0.12	17.50 \pm 4.79	1.72 \pm 0.33
PMN-E (μ g/ml)	0.08 \pm 0.02	0.50 \pm 0.14	0.11 \pm 0.02	0.45 \pm 0.09	0.14 \pm 0.02
MPO (μ g/ml)	0.13 \pm 0.02	1.93 \pm 0.56	0.15 \pm 0.02	2.73 \pm 0.78	0.29 \pm 0.07
Lys (μ g/ml)	0.13 \pm 0.03	2.45 \pm 0.89	0.12 \pm 0.03	0.79 \pm 0.17	0.19 \pm 0.05

UC was defined as being in the active phase (UC-A) if the colonoscopic grade of the patient was \geq 2. CD was defined as being in the active phase (CD-A) if the CDAI score of the patient was $>$ 150. Values are expressed as means \pm SEMs. CD-I = CD (inactive phase); UC-I = UC (inactive phase).

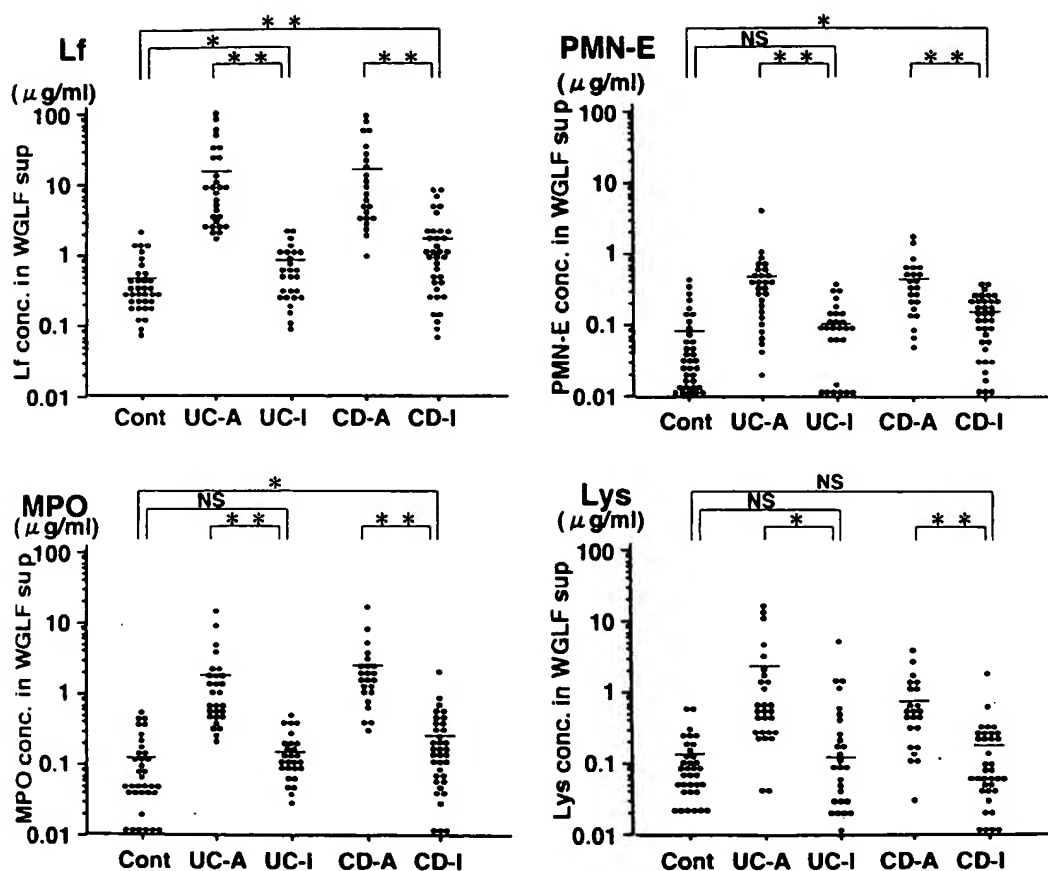


Figure 2. Concentrations of lactoferrin (Lf), PMN-E, myeloperoxidase (MPO), and lysozyme (Lys) in the WGLF supernatant (supernatant after centrifugation) in UC and CD. The concentrations were expressed on a logarithmic scale. UC was defined as being in the active phase (UC-A) if the colonoscopic grade of the patient was ≥ 2 . CD was defined as being in the active phase (CD-A) if the CDAI score of the patient was >150 . CD-I = CD (inactive phase); cont = control; NS = nonsignificant; UC-I = UC (inactive phase). * $p < 0.05$, Student's t test. ** $p < 0.01$, Student's t test.

not high in zero (0%), 12 (42.9%), five (17.9%), and 10 (35.7%) of the 28 samples, respectively. In inactive UC (grade 1), lactoferrin, PMN-E, myeloperoxidase, and lysozyme were high in three (11.1%), one (3.7%), one (3.7%), and three (11.1%) of the 27 samples, respectively. All active UC patients (grade ≥ 2) showed high concentrations of lactoferrin, indicating that lactoferrin in WGLF did not overlook minimal inflammation remaining in the colonic mucosa. The relationship between concentrations in total WGLF and colonoscopic grading was similar to that between concentrations in the WGLF supernatant and colono-

scopic grading. The correlation coefficients between colonoscopic grading and lactoferrin, PMN-E, myeloperoxidase, and lysozyme in total WGLF were 0.887, 0.541, 0.843, and 0.654, respectively.

Relationship Between Protein Concentrations in WGLF and the CDAI in CD

As shown in Figure 5, lactoferrin and myeloperoxidase concentrations in the WGLF supernatant showed good correlations with the CDAI. The correlation coefficients between the CDAI and lactoferrin, PMN-E, myeloperoxidase,

Table 2. Concentrations of Lactoferrin (Lf), PMN-E, Myeloperoxidase (MPC), and Lysozyme (Lys) in the Total WGLF (Without Centrifugation) in UC and CD

	Control (n = 35)	UC-A (n = 28)	UC-I (n = 27)	CD-A (n = 23)	CD-I (n = 36)
Lf ($\mu\text{g/ml}$)	0.89 ± 0.18	37.14 ± 11.76	1.31 ± 0.19	28.59 ± 7.64	3.61 ± 0.85
PMN-E ($\mu\text{g/ml}$)	0.18 ± 0.04	1.31 ± 0.28	0.37 ± 0.06	0.89 ± 0.15	0.40 ± 0.05
MPO ($\mu\text{g/ml}$)	0.26 ± 0.05	9.32 ± 3.00	0.44 ± 0.08	9.02 ± 2.11	1.09 ± 0.23
Lys ($\mu\text{g/ml}$)	0.34 ± 0.10	9.24 ± 3.33	1.17 ± 0.35	4.24 ± 1.05	0.82 ± 0.20

UC was defined as being in the active phase (UC-A) if the colonoscopic grade of the patient was ≥ 2 . CD was defined as being in the active phase (CD-A) if the CDAI score of the patient was >150 . Values are expressed as means \pm SEMs. CD-I = CD (inactive phase); UC-I = UC (inactive phase).

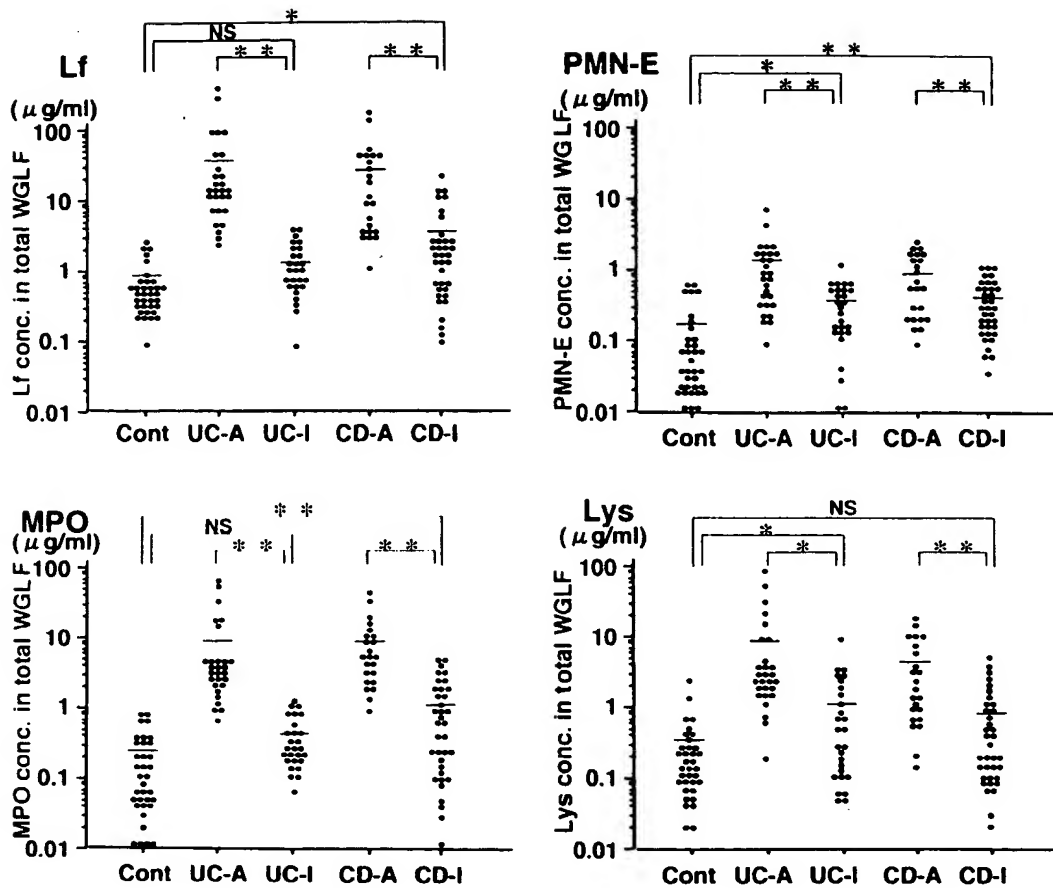


Figure 3. Concentrations of lactoferrin (Lf), PMN-E, myeloperoxidase (MPO), and lysozyme (Lys) in total WGLF (without centrifugation) in UC and CD. The concentrations were expressed on a logarithmic scale. UC was defined as being in the active phase (UC-A) if the colonoscopic grade of the patient was ≥ 2 . CD was defined as being in the active phase (CD-A) if the CDAI score of the patient was >150 . CD-I, Crohn's disease (inactive phase); cont = control; NS = nonsignificant; UC-I = ulcerative colitis (inactive phase). * $p < 0.05$, Student's t test. ** $p < 0.01$, Student's t test.

and lysozyme in the WGLF supernatant were 0.856, 0.438, 0.844, and 0.573, respectively. In active CD (CDAI >150), lactoferrin, PMN-E, myeloperoxidase, and lysozyme were not high in one (4.3%), 12 (52.2%), one (4.3%), and nine (39.1%) of the 23 samples, respectively. In inactive CD (CDAI ≤ 150), lactoferrin, PMN-E, myeloperoxidase, and lysozyme were high in 13 (36.1%), zero (0%), seven (19.4%), and two (5.6%) of the 36 samples, respectively. The relationship between concentrations in total WGLF and CDAI was similar to that between concentrations in the WGLF supernatant and CDAI. The correlation coefficients between CDAI and lactoferrin, PMN-E, myeloperoxidase, and lysozyme in the total WGLF were 0.805, 0.408, 0.779, and 0.622, respectively.

Supernatant/Total WGLF Ratio (S/T Ratio) of Neutrophil-Derived Proteins (Lactoferrin, PMN-E, Myeloperoxidase, and Lysozyme) in UC and CD

The values are shown in Figure 6. The S/T ratio is the ratio of the concentration in the WGLF supernatant (supernatant after centrifugation) and that in the total WGLF (without

centrifugation). The S/T ratio represents the proportion of neutrophil-derived protein released from neutrophils. The S/T ratio of lactoferrin was the highest of these four proteins in all disease groups and control subjects. Interestingly, in active UC patients, in whom neutrophils are believed to strongly participate in the disease pathophysiology, the S/T ratio was lower than that in inactive UC or control subjects.

Conventional Laboratory Indices of Disease Activity and the Concentrations of Neutrophil-Derived Proteins (Lactoferrin, PMN-E, Myeloperoxidase, and Lysozyme) in WGLF

The positivity rates are shown in Table 3. The white blood cell (WBC) count, platelet count, and serum C-reactive protein (CRP) level were not sensitive markers to discriminate between active and inactive phases in either UC or CD. In UC, the correlation coefficients between colonoscopic grading and WBC count, platelet count, and serum CRP level were 0.307, 0.324, and 0.493, respectively. In CD, the correlation coefficients between the CDAI and WBC count,

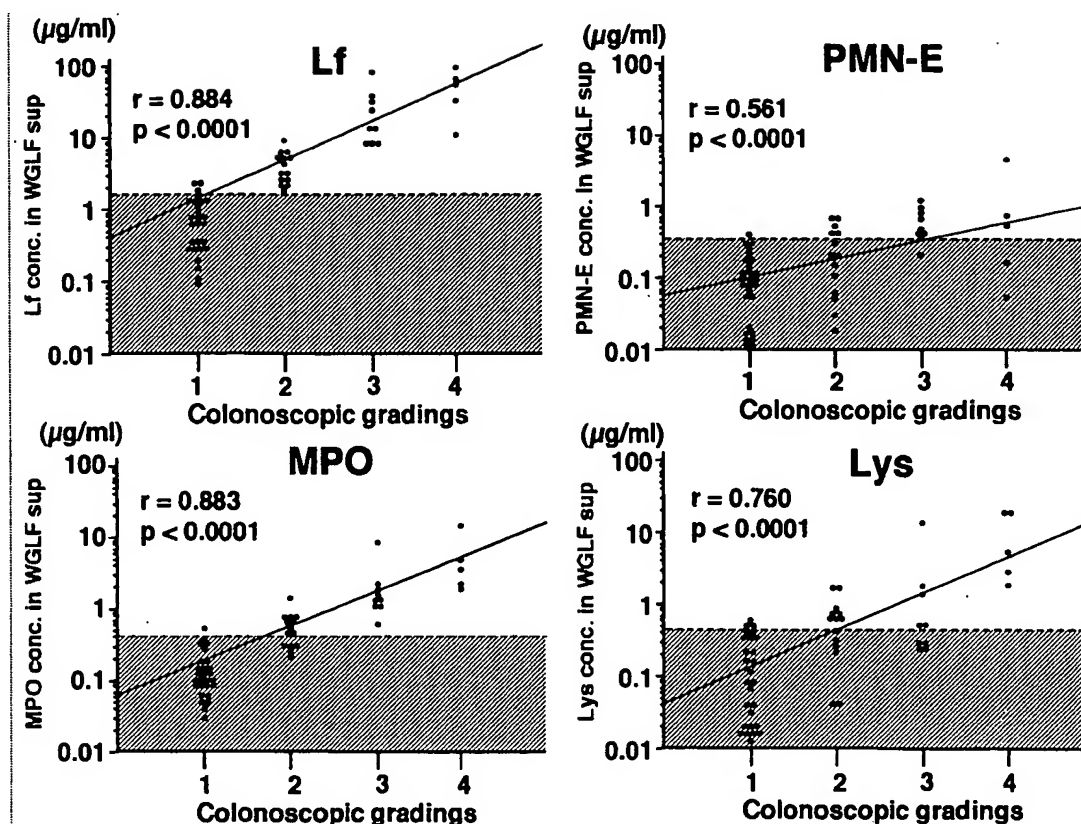


Figure 4. Concentrations of neutrophil-derived proteins (lactoferrin [Lf], PMN-E, myeloperoxidase [MPO], and lysozyme [Lys]) in WGLF supernatants and the colonoscopic grading in UC. The concentrations were expressed on a logarithmic scale. The colonoscopic degree of inflammation was graded according to the criteria described in Materials and Methods. Dotted lines indicate the means + 2 SDs of the control subjects (1.58, 0.34, 0.41, and 0.45 µg/ml for lactoferrin, PMN-E, myeloperoxidase, and lysozyme, respectively).

platelet count, and serum CRP level were 0.419, 0.184, and 0.663, respectively.

The positivity rate of lactoferrin was the highest of these four proteins in WGLF supernatant in both active UC and active CD. In active UC, the positive predictive value and the negative predictive value of lactoferrin in WGLF supernatant were 90.3% and 100%, respectively. In active CD, the positive predictive value and the negative predictive value of lactoferrin in WGLF supernatant were 88.0% and 97.0%, respectively. ROC analysis was also performed using protein concentration in WGLF supernatant of active IBD patients and control subjects. The areas under ROC curves of lactoferrin, PMN-E, myeloperoxidase, and lysozyme in active UC were 0.990 ± 0.008 (area under ROC curve \pm SEM), 0.876 ± 0.043 , 0.966 ± 0.018 , and 0.898 ± 0.045 , respectively. Respective values in active CD were 0.993 ± 0.007 , 0.899 ± 0.039 , 0.992 ± 0.008 , and 0.888 ± 0.047 .

DISCUSSION

The methylene blue method has been used for counting fecal leukocytes (22). However, this method requires fresh stool samples and the immediate availability of a person skilled in

the use of the technique. Leukocyte scintigraphy using labeled granulocytes is another method for assessing fecal leukocytes (23, 24), but it is expensive and cannot be performed serially in individual patients because of exposure problems. Therefore, these methods cannot be widely used clinically. We previously reported that fecal neutrophil-derived proteins were useful markers for disease activity in IBD (15, 17). Handy *et al.* (25) reported that the neutrophil count in WGLF is useful for evaluating neutrophil infiltration in the intestinal mucosa in patients with IBD. They also found that measurement of PMN-E in WGLF is useful for evaluating neutrophil infiltration in the mucosa (12). Neutrophil-derived proteins other than PMN-E, however, have not yet been studied. Also, the release of these proteins from neutrophils in WGLF and their stability in WGLF have not been investigated. For the neutrophil-derived proteins in WGLF to sensitively reflect intestinal inflammation, it is preferable that they should be released efficiently from neutrophils and be stable in the lavage fluid.

In the present study, we focused on lactoferrin, PMN-E, myeloperoxidase, and lysozyme in WGLF. Lactoferrin is found in specific neutrophil granules, PMN-E and myeloperoxidase are found in the azurophilic granules, and lysozyme is found in both types of granules (26, 27). These

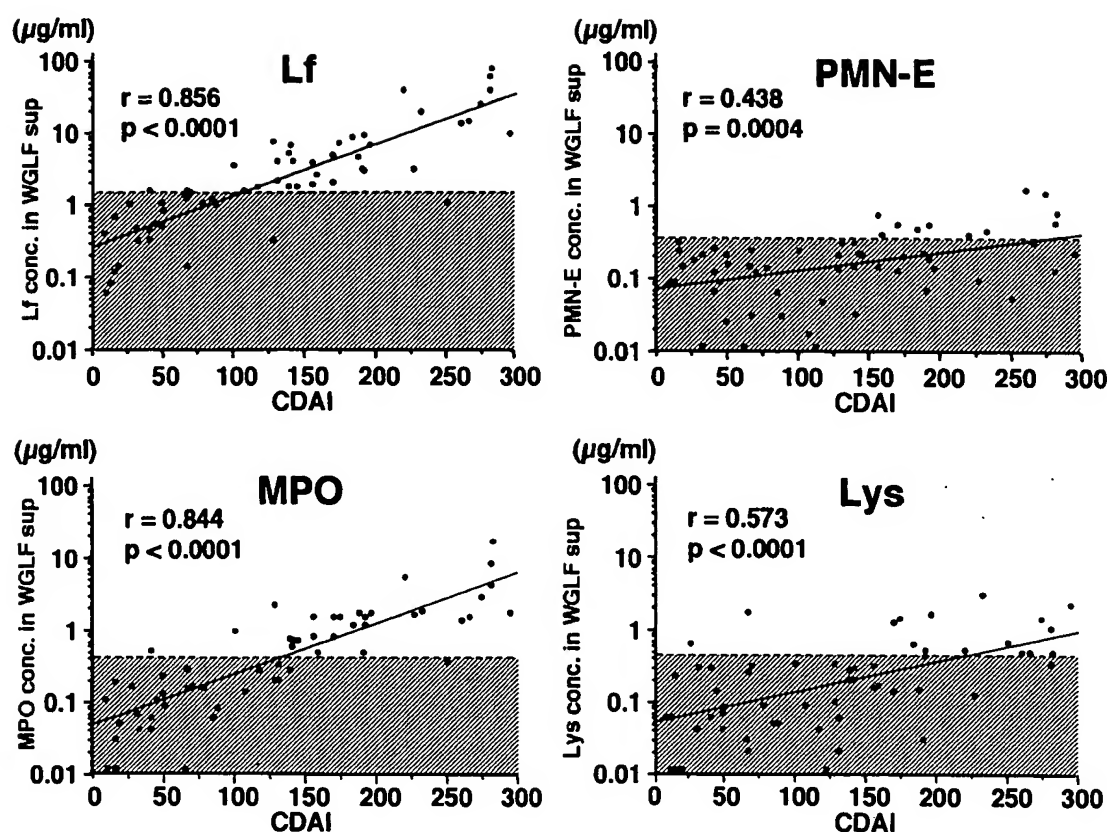


Figure 5. Concentrations of neutrophil-derived proteins (lactoferrin [Lf], PMN-E, myeloperoxidase [MPO], and lysozyme [Lys]) in WGLF supernatants and the CDAI score in CD. The concentrations were expressed on a logarithmic scale. Dotted lines show the means + 2 SDs of the control subjects (1.58, 0.34, 0.41, and 0.45 µg/ml for lactoferrin, PMN-E, myeloperoxidase, and lysozyme, respectively).

neutrophil-granular proteins have been proposed as target antigens of antineutrophil cytoplasmic antibodies (28, 29). Lactoferrin, an iron-binding protein with a molecular weight of approximately 80,000, is found in various secretions, such as breast milk, tears, and saliva, as well as in specific neutrophil granules. Lactoferrin is present in the intestinal mucus and has an antibacterial effect (30, 31). PMN-E is present in plasma as an elastase/ α_1 -antitrypsin complex. Recently, we demonstrated that most PMN-E was not a complex with α_1 -antitrypsin in feces (15). Myeloperoxidase activity in the mucosa has been used as an index of neutrophil infiltration in experimental colitis models (32). Lysozyme is contained not only in neutrophils, but also in macrophages, Brunner's glands, and Paneth cells. These neutrophil-granular proteins are released extracellularly when the cell is activated.

In patients with UC, the lactoferrin concentration in WGLF had the best correlation with the colonoscopic grading. The lactoferrin concentration was high in all active UC patients (grade ≥ 2). In contrast, PMN-E and lysozyme concentrations were not high in a considerable number of active UC patients (grade ≥ 2). These findings seem to be partly attributable to the different stabilities of these proteins in WGLF. Our results indicated that lactoferrin is the most

suitable marker of the four for evaluating mucosal inflammation in UC. In patients with CD, lactoferrin and myeloperoxidase concentrations in WGLF correlated well with the CDAI. Lactoferrin and myeloperoxidase were high in 22 of 23 active CD patients (CDAI > 150). However, these two proteins were high in 13 (36.1%) and seven (19.4%), respectively, of the 36 inactive CD patients (CDAI ≤ 150). In 11 of 13 (84.6%) and six of seven (85.7%) of these patients, ulceration was found by colonoscopy or small bowel x-ray. These findings suggest that lactoferrin and myeloperoxidase in WGLF are excellent markers for evaluating the disease activity of CD. Lactoferrin has a further advantage as a marker because a larger amount of lactoferrin is contained in WGLF than of the other three proteins. In daily medical practice, a high WBC count, an increased platelet count, and an elevated serum CRP level are generally considered to indicate that the IBD is in an active stage. As shown in Table 3, however, these parameters are not sensitive for distinguishing between active and inactive disease. It is commonly observed that mucosal inflammation persists even after the symptoms disappear and conventional laboratory indices return to normal. Even if colonoscopy is not performed, inflammation of the intestinal mucosa can be evaluated by obtaining WGLF and measuring the lactoferrin

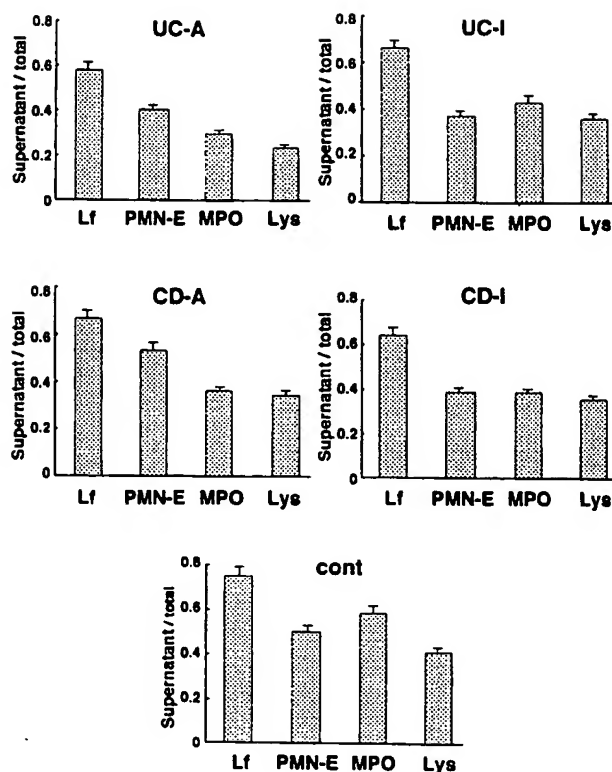


Figure 6. Supernatant/total WGLF ratio of neutrophil-derived proteins (lactoferrin [Lf], PMN-E, myeloperoxidase [MPO], and lysozyme [Lys]) in UC and CD. The supernatant/total WGLF ratio of each sample indicates the ratio of the concentration in the WGLF supernatant (supernatant after centrifugation) and that in total WGLF (without centrifugation). UC was defined as being in the active phase (UC-A) if the colonoscopic grade of the patient was ≥ 2 . CD was defined as being in the active phase (CD-A) if the CDAI score of the patient was >150 . Values are expressed as means \pm SEMs. CD-I, Crohn's disease (inactive phase); cont = control; UC-I, ulcerative colitis (inactive phase).

concentration. However, it should be kept in mind that a whole gut lavage might exacerbate the disease in patients with severe inflammation.

Lesions of the small intestine are difficult to detect compared to colonic lesions because of the limited availability of endoscopy. We assumed that lactoferrin in WGLF is useful

for detecting lesions in the small intestine. In the present study, lactoferrin concentrations were high in 10 of 12 CD patients (83.3%) who had active disease in the small intestine but not in the large intestine. This finding suggests that the small intestine can be evaluated by WGLF. When colonoscopy fails to detect any lesions, a WGLF sample may be useful to evaluate the small intestine. A high concentration suggests involvement of the small intestine or the more proximal digestive tract. Lavage fluid obtained from the terminal ileum would presumably allow more accurate evaluation of the small intestine than lavage fluid from the rectum.

We previously reported on fecal concentrations and daily fecal excretion of neutrophil-derived proteins. The fecal concentrations of all of the neutrophil-derived proteins were significantly higher in patients with active disease than in the inactive phase or in control subjects. Of the fecal markers, lactoferrin was the most useful for UC, and lactoferrin and myeloperoxidase were best for CD. However, colonoscopy was not performed in all patients in our previous study. In the present study, colonoscopy was performed in all patients. Fecal lactoferrin excretions (mg/day) were 0.21 ± 0.05 , 50.2 ± 5.5 , and 34.3 ± 6.7 in control, active UC, and active CD subjects, respectively (17). Loss of protein into the gut lumen, which may approximate to fecal excretion, can be estimated by the concentration in WGLF (9). The rate of fluid passage along the gut during lavage is approximately 1 L/h (0.017 L/min), so the estimated daily loss (mg/day) of a certain substance can be obtained by the following equation: $24 \text{ (L)} \times \text{the concentration in WGLF (mg/L)}$. The present study showed that the estimated daily lactoferrin losses (mg/day) into the gut lumen were 21.6 ± 4.8 , 890.4 ± 283.2 , and 686.4 ± 182.4 in control, active UC, and active CD subjects, respectively. The daily lactoferrin output estimated from WGLF was much larger than that from feces. Neutrophils and lactoferrin in the gut lumen are recovered immediately by lavage fluid. Otherwise, neutrophils and lactoferrin remain for a while when protein is degenerated by digestive enzymes and bacterial proteases. The number of neutrophils that migrate into the gut lumen is very likely much larger than first thought.

In the present study, neutrophil transmigration and de-

Table 3. Positivity Rates of Conventional Laboratory Indices of Disease Activity and the Concentrations of Neutrophil-Derived Proteins (Lactoferrin [Lf], PMN-E, Myeloperoxidase [MPO], and Lysozyme [Lys]) in WGLF

		UC		CD	
		Active	Inactive	Active	Inactive
WBC	$>8,190/\mu\text{l}$	5/26 (19.2%)	0/27 (0.0%)	8/23 (34.8%)	3/26 (11.5%)
Platelet count	$>329,000/\mu\text{l}$	5/26 (19.2%)	0/27 (0.0%)	10/23 (43.5%)	3/26 (11.5%)
CRP	$>0.25 \text{ mg/dl}$	11/26 (42.3%)	1/27 (3.7%)	20/23 (87.9%)	3/26 (11.5%)
Lf in WGLF*	$>1.58 \mu\text{g/ml}$	28/28 (100%)	3/27 (11.1%)	22/23 (95.7%)	13/36 (36.1%)
PMN-E in WGLF*	$>0.34 \mu\text{g/ml}$	16/28 (57.1%)	1/27 (3.7%)	11/23 (47.8%)	0/36 (0.0%)
MPO in WGLF*	$>0.41 \mu\text{g/ml}$	23/28 (82.1%)	1/27 (3.7%)	22/23 (95.7%)	7/36 (19.4%)
Lys in WGLF*	$>0.45 \mu\text{g/ml}$	18/28 (64.3%)	3/27 (11.1%)	14/23 (60.9%)	2/36 (5.6%)

* Concentration in WGLF supernatant (supernatant after centrifugation).

granulation were studied by comparing the neutrophil-derived protein concentration in a supernatant with that in total WGLF. Presumably, the supernatant contains proteins that are released by neutrophils in the intestinal mucosa as well as in the gut lumen. As shown in Figure 6, lactoferrin was the most efficiently released from neutrophils among the neutrophil-derived proteins we examined. Our results also indicate that not all neutrophils undergo degranulation after migrating into the gut lumen, resulting in a considerable number of neutrophils being excreted in feces without degranulation. In active UC patients, the S/T ratio of these proteins was not higher than those in other disease groups. However, considering that the total amount of these proteins released into the gut lumen was much larger in active UC patients, our findings suggest that neutrophils or neutrophil-granular proteins play an important role in the pathophysiology of the disease.

PEG has been used as a nonabsorbable marker in intestinal perfusion studies. Patients with more extensive inflammation might be in a secretory state and would dilute protein concentrations. Therefore, it would be useful to normalize the protein concentration against effluent PEG concentration: by normalization against effluent PEG concentration, the difference in protein concentration in WGLF between active and inactive phases might become more distinct. Although protein concentrations in WGLF were not normalized in the present study, the concentrations of neutrophil-derived proteins in WGLF were significantly higher in the active phase than in the inactive phase and control subjects.

In conclusion, the assay of neutrophil-derived proteins in WGLF is an objective means of grading mucosal disease activity in patients with IBD. This method may be useful not only in a research setting, but also in a clinical setting. Lactoferrin is the most suitable of these proteins for use as a neutrophil-derived WGLF marker of intestinal inflammation.

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Serologic testing for inflammatory bowel disease

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Objectives: To determine the accuracy of anti-neutrophil cytoplasmic antibodies (ANCAs) and anti-*Saccharomyces cerevisiae* antibodies (ASCA) in distinguishing patients with inflammatory bowel disease from patients with other disorders, seen in a pediatric gastroenterology clinic setting, and in distinguishing ulcerative colitis (UC) from Crohn's disease (CD).

Study design: Serum samples from 120 children with new or established diagnoses of UC (n = 25) or CD (n = 20) and control children (n = 74) were analyzed in blinded fashion for the presence of IgG ANCAs and IgA and IgG ASCA.

Results: The highest sensitivity for detecting inflammatory bowel disease, 71%, was achieved by using ANCAs and ASCA together. The best test for UC was ANCAs, which had a sensitivity of 80%. However, the ANCA pattern characteristic of UC, perinuclear ANCAs eliminated by DNase, had a sensitivity of 60%. High-titer ANCAs were specific for UC, whereas ASCA were specific for CD.

Conclusions: Testing for ANCAs and ASCA together did not achieve sensitivity necessary for population screening. However, ANCAs and ASCA may be helpful in evaluating children suspected of having inflammatory bowel disease and in distinguishing UC from CD. (J Pediatr 1999;134:447-52)

Inflammatory bowel disease is characterized by chronic gastrointestinal tract inflammation of unknown etiology. Immunologic, environmental, infectious, and genetic factors have been postulated to increase the risk for developing IBD.¹

Signs and symptoms of IBD are often nonspecific, and it is often a clinical challenge to distinguish IBD from other disorders of childhood and adolescence such as chronic diarrhea, recurrent abdominal pain, acute infectious colitis, anorexia, and short

stature. In addition, the onset of IBD may be preceded by extra-intestinal manifestations, such as hepatitis (sclerosing cholangitis, autoimmune hepatitis) or arthritis² without diarrhea or rectal bleeding.³ Currently, the diagnosis of IBD requires a combination of typical clinical signs and symptoms, exclusion of other disorders, plus radiographic, endoscopic, and histologic features consistent with IBD.⁴ Despite careful clinical evaluation, 10% to 15% of children with colitis have an indeterminate form,^{5,6} and 2% to 4% of patients undergoing colectomy with ileoanal anastomosis for treatment of ulcerative colitis are ultimately determined to have Crohn's disease.^{7,8} Therefore readily available, noninvasive tests are needed to make a timely and accurate diagnosis of UC or CD.

ANCAs	Anti-neutrophil cytoplasmic antibodies
ASCAs	Anti- <i>Saccharomyces cerevisiae</i> antibodies
CD	Crohn's disease
ELISA	Enzyme-linked immunosorbent assay
EU	ELISA units
IBD	Inflammatory bowel disease
pANCAs	Perinuclear ANCAs
UC	Ulcerative colitis

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Recently, serologic tests for IBD have become commercially available. Anti-neutrophil cytoplasmic antibodies are detected in up to 60% to 70% of adults and children with UC.⁹⁻¹¹ Up to 60% to 70% of adults with CD have circulating anti-*Saccharomyces cerevisiae* antibodies.¹² The significance of these antibodies remains unclear. Titers of pANCAs do not change with disease activity but may predict treatment-resistant colitis and pouchitis.^{13,14} The

Table 1. Demographic data in children with UC, CD, and non-IBD disorders (control group) seen in an outpatient setting

	UC group (n = 25)	CD group (n = 20)	Control group (n = 74)	P value
Male (%)	36	70	46	.07
Caucasian (%)	79	94	83	.35
Mean age (y)	13.6	15.3	10.7	.0001*
SD	2.9	2.6	4.5	
Median	13.0	15.5	11.0	
Range	8-20	11-20	0.2-25	

*Student-Newman-Keuls post-analysis of variance test: control versus UC, $P < .05$; control versus CD, $P < .05$.

presence of perinuclear ANCA is evidence for a class II major histocompatibility complex-driven T-cell activation and thus a possible genetic predisposition to develop UC.¹⁵ IgA and IgG ASCAs have been associated with small bowel involvement.¹⁶

The purpose of this study was to evaluate the accuracy of ANCA and ASCAs in identifying IBD and to differentiate UC from CD in children seen in a pediatric gastroenterology clinic.

METHODS

Study Population and Serum Samples

All children were evaluated in an academic pediatric gastroenterology clinic in Denver, Colorado, between July 1992 and May 1997. Children were recruited if they were having blood drawn for other reasons or were having an intravenous line placed for an endoscopic procedure. Children were evaluated at times variable from the time of clinical diagnosis of IBD or other disease. Some children were undergoing initial evaluation, and others with established IBD or other diagnoses had ongoing treatment or follow-up. Control children were included if their symptoms or diagnosis might be related to IBD (eg, abdominal pain, failure to thrive, or chronic diarrhea). Children with liver disease were included because of the association of sclerosing cholangitis and autoimmune

hepatitis with IBD and because of a possible association of ANCA with pediatric liver disease, specifically biliary atresia.¹⁷ Signed informed consent was obtained at the time of a blood draw. Serum samples were stored at -70°C until sent for analysis to Prometheus Laboratories (San Diego, Calif). Analysis was performed without knowledge of the clinical history or diagnosis of the patient. Clinical data were obtained from the medical records. Diagnosis of UC or CD was made by published clinical, radiographic, endoscopic, and histologic criteria.^{4,5} In all patients with IBD, the clinical features over time allowed for the diagnosis of UC or CD; no patient was classified as having indeterminate colitis. UC was characterized by chronic inflammation of the colonic mucosa, in a continuous distribution, extending a variable distance from the rectum. CD was characterized by chronic inflammation in any location throughout the gastrointestinal tract in the absence of another cause, frequently with segmental involvement and normal intervening areas (skip areas). Pathognomonic features of CD included jejunal or ileal involvement, skip areas, serpiginous ulcers, and the presence of granulomas.^{1,4,5} Gastroduodenitis was not specific for UC or CD.¹⁸

This study was approved by the Colorado Multiple Institutional Review Board and The Children's Hospital, Denver.

ANCA

The Prometheus UC Diagnostic system, UC•Dx1, is a 3-step process that detects and quantitates ANCA in serum as reported by Winter et al⁹ and by Ruemmele et al.¹⁹ First, ANCA IgG antibody levels are quantitated in a microplate enzyme-linked immunosorbent assay relative to reference sera. Second, positive specimens are evaluated by an indirect immunofluorescence assay, which detects a perinuclear pattern, pANCA. Specimens with perinuclear patterns undergo a third step, DNase treatment. Elimination of pANCA by DNase is noted in 60% to 80% of patients with UC, whereas pANCA retention is rare, noted in 3% of patients with UC. ANCA were considered positive if above 8.9 to 13.8 ELISA units per milliliter (EU/mL), reflecting run-to-run differences with normal non-UC reference sera from healthy volunteers.

ASCA

In the Prometheus CD•Dx1 assay, circulating anti-oligosaccharide IgA and IgG ASCAs were detected by using microtiter plates coated with phosphopeptidomannans from *Saccharomyces uvarum*, a yeast, as reported by Ruemmele et al.¹⁹ Bound antibodies from samples diluted 1:100 were labeled with alkaline phosphatase-conjugated goat anti-human IgG. Specific absorbance was measured at 405 nm after addition of *p*-nitrophenol. ELISA units were assigned relative to a Prometheus Laboratory standard, arbitrarily designated as 100 EU/mL. ASCAs were considered positive if above 20 EU/mL for IgA and above 40 EU/mL for IgG.

Statistical Analysis

Categorical variables were analyzed by using a χ^2 test. Continuous variables were analyzed by using analysis of variance with the appropriate adjustment if the assumption of equal variances was not met. Post-analysis of variance comparisons were performed by using the Student-Newman-Keuls test.

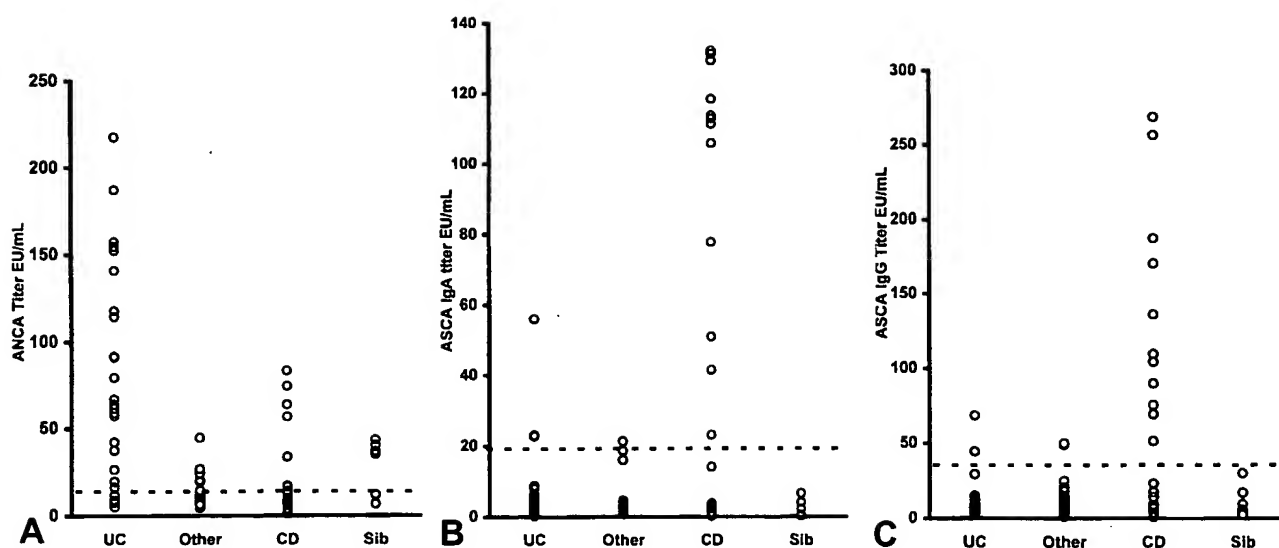


Figure 1. A, Quantitation of IgG ANCA with an ELISA for evaluation of children with UC (n = 25), CD (n = 20), and non-IBD disorders (control, n = 74) and 6 healthy siblings of children with IBD. Although ANCA were commonly detected in children with UC and CD, only patients with UC had ANCA titers >1:100. B and C, Quantitation of IgA and IgG ASCAs with an ELISA for evaluation of children with UC (n = 25), CD (n = 20), and non-IBD disorders (control, n = 74) and 6 healthy siblings of children with IBD. Most children with positive ASCA titers had CD and had both IgA and IgG antibodies.

Sensitivity, specificity, and predictive values were calculated to evaluate the capability of the antibody titers to predict IBD. The diagnostic tests were evaluated first as dichotomous variables in which the recommended cut-off for adults was used to define the test result as positive or negative. Second, the predictive value of the antibody titers as continuous and as categorical variables was further evaluated by using logistic regression models. The goodness of fit of the logistic regression models was compared by the Akaike's information criterion, which is a variation of the -2 log likelihood method.²⁰

RESULTS

A total of 120 children were evaluated, including 26 with UC, 20 with CD, and 74 non-IBD control children (Table 1). The groups were comparable in terms of gender and ethnic distribution. However, the children with IBD were older than the control children, consistent with the peak age of

Table 1. ANCA and ASCA patterns in children with UC, CD, and non-IBD disorders (control), seen in an outpatient setting, and in healthy siblings of patients with IBD

ANCA pattern	UC group (n = 25)	CD group (n = 20)	Siblings (n = 6)	Control group (n = 74)
Perinuclear				
DNase resistant	3	0	0	0
DNase eliminated	10	7	0	4
Cytoplasmic	2	1	0	1
Perinuclear and cytoplasmic				
DNase resistant	1	0	0	1
DNase eliminated	4	0	4	2
None	5	12	2	66
Total	25	20	6	74
ASCA				
IgA	1	1	0	1
IgG	0	0	0	2
IgA + IgG	2	11	0	1
None	22	8	6	70
Total	25	20	6	74

onset of IBD. The 74 children in the control group included 33 with inflammation of the upper gastrointestinal tract: *Helicobacter pylori* gastritis (n = 4); celiac disease (n = 3); and inflamma-

tion of the upper gastrointestinal tract from gastroesophageal reflux, allergic enteropathy, and nonspecific gastro-duodenitis (n = 26). The remaining 41 control children had the following di-

Table III. Predictive power of ANCAs and ASCAs to distinguish children with IBD (n = 45) from those with non-IBD disorders (n = 74)

	ANCAs	ASCAs	Combined
Sensitivity (%)	49	33	71
Specificity (%)	91	95	84
Positive predictive value (%)	76	79	73
Negative predictive value (%)	75	71	83

For identifying children with IBD, the highest sensitivity and specificity were obtained with ANCA and ASCA testing combined.

Table IV. Predictive power of pANCAs and ASCAs to discriminate UC (n = 25) from CD (n = 20)

	pANCAs* for UC	ASCAs for CD
Sensitivity (%)	60	60
Specificity (%)	65	88
Positive predictive value (%)	68	80
Negative predictive value (%)	56	73

*For this analysis, pANCAs eliminated by DNase, the pattern typical of UC, was used.

agnoses: functional abdominal pain (n = 21), colonic polyps causing rectal bleeding (n = 3), self-limited idiopathic acute colitis (n = 1), idiopathic peritonitis (n = 1), constipation (n = 1), intestinal graft-versus-host disease after bone marrow transplantation (n = 1), liver disease (n = 6, 1 each with sickle cell disease, liver tumor, cholelithiasis, progressive familial intrahepatic cholestasis, autoimmune hepatitis, and acute idiopathic self-limited hepatitis), and no disease (n = 7).

Of the 25 children with UC, 80% had detectable ANCAs (Figure, A). In contrast, ANCAs were detected in 40% of children with CD and 11% of control children without IBD. The ANCA pattern most common in UC, pANCAs eliminated by DNase treatment, was identified in 15 (60%) children with UC (Table II). Five of these 15 children with UC and positive pANCAs also had cytoplasmic ANCAs (c+pANCAs). Five (20%) children with UC had no detectable ANCAs.

The titer of ANCAs in UC overlaps with that in CD (Figure, A). However, titers above 100 EU/mL were specific for UC.

Testing was also performed in 6 healthy siblings of children with IBD (5 with CD and 1 with UC). ANCAs were detected in 4 (67%). None of the siblings had signs or symptoms of IBD.

Positive titers of ASCA IgA and IgG were found in 12% of children with UC, 60% of children with CD, and 4% of control children (Figure, B and C). The most common pattern, both IgA and IgG ASCAs present, was noted in 2 of 3 children with UC, 11 of 12 children with CD, and 2 of 3 control children with positive ASCA titers.

Both ANCAs and ASCAs were identified in 9 children (3 with UC, 4 with CD, and 2 control). The most common pattern, pANCAs eliminated by DNase plus both IgA and IgG ASCAs, was seen in 5 children. The other 4 children had 3 different patterns.

Table III shows the predictive power of ANCAs and ASCAs individually, or combined, in identifying children with IBD from control children without IBD. The best accuracy was obtained by combining results of the 2 tests. The highest specificity (95%) was provided by ASCAs. In a logistic regression analysis, the ANCA assay was the sin-

gle best test with which to distinguish children with IBD from those without IBD. Use of antibody titers as continuous variables in a logistic regression model did not significantly improve the predictive power over the model in which the traditional categorical (positive/negative) variables were used.

Table IV shows the predictive power of each test for identifying the type of IBD. The ANCA pattern characteristic of UC, pANCAs eliminated by DNase, had a sensitivity of 60% and a specificity of 65%. Similarly, the ASCA test for CD also had a sensitivity of 60% but had a higher specificity, 88%.

DISCUSSION

Our data suggest that testing for ANCAs together with ASCAs identifies the majority of children with IBD but does not have an accuracy useful for population screening. The presence of these antibodies may be helpful in confirming the clinical suspicion of IBD. Earlier diagnosis may decrease the long-term morbidity of IBD, which includes delayed puberty and shorter ultimate height,²¹ diminished bone density,²² vitamin and mineral deficiencies,²³ and psychologic adjustment to chronic illness.²⁴

Several differences in methodology and study design limit direct comparison of our study with 4 other case-control studies evaluating ANCAs and/or ASCAs in children.^{9,11,19,25} First, the ANCA assays are not standardized, and the DNase specificity step was used in only one other study.¹⁹ Second, the prevalence of IBD in the study group was quite different among the different studies. This is important in assessing the accuracy of the serologic tests because the positive predictive value tends to increase as the disease prevalence increases.²⁶ Compared with our study population, that of Ruemmele et al¹⁹ had a higher prevalence of IBD (37% vs 70%) and of CD (17% vs 50%) and showed a higher positive predictive value of combined

ASCA and ANCA testing for IBD (73% vs 96%) and of ASCA for CD (80% vs 92%). In contrast, our study population had a higher prevalence of UC (20% vs 14%), and we found a higher positive predictive value of pANCA for UC (68% vs 54%). A third limitation in comparing studies is that the control groups were quite variable, consisting of a mixture of healthy children, siblings, and control subjects without IBD. In addition, there were large differences between the mean ages of the disease group and the control group. For example, although the IBD groups generally had a mean age between 12 and 15 years, the control group had a much younger mean age in some studies—5.9 years⁹ and 6.5 years.¹⁹ The mean age of the control group in our study was 10.7 years, closer to that of the IBD group. The age of the control group is important because the age at which ANCAs and ASCAs become detectable is unknown. A fourth and long-standing problem has been the absence of a "gold standard" for defining IBD and for discriminating UC from CD. Finally, other factors, such as genetic differences between the populations studied, might affect the accuracy of these tests. Therefore large prospective studies are needed to assess the accuracy and utility of serologic testing in different settings and in different populations.

The serologic assays may have been affected by the variable times after the diagnosis of IBD and other conditions. This is unlikely for several reasons. Our data are in agreement with other studies showing that ASCAs are detected in about 60% of children¹⁹ and adults²⁷ with CD. Furthermore, the titer of pANCA is independent of disease activity and medical and surgical therapy and persists after colectomy.^{16,19,28-30} Similarly, ASCAs are independent of disease activity, disease duration, and medical treatment.¹⁹ Thus the validity of the ANCA and ASCA assays is unlikely to be affected by variable disease duration and treatment.

Our findings that 80% of children with UC have ANCAs and that 60% have pANCA eliminated by DNase are consistent with previous reports.^{9,11,19,25} Our data confirm a previous observation that high-titer ANCAs are specific for UC. Similarly, our finding that 40% of children with CD have ANCAs is consistent with reports in children¹⁹ and adults.³¹ Although pANCA have been associated with a "UC-like" subtype of CD, we were unable to assess this association because almost all the children with CD in our study had colitis.³¹

Future studies will need to address 2 issues. One issue is the determination of whether serologic markers such as ANCAs and ASCAs provide clinically useful information, such as disease location, disease severity, likelihood of specific complications, and identification of UC or CD in patients with indeterminate colitis. This type of information may provide a rationale for choosing specific therapies for treatment and prevention of complications associated with IBD. The large number of patients needed to assess the clinical utility of serologic testing is beyond the scope of this study.

Another issue is the development of tools for screening general populations and groups at high risk for IBD. Healthy first-degree relatives of patients with IBD have a high frequency of ASCAs (20%)³² and of ANCAs (25%).^{11,15} Thus antibodies associated with IBD may be commonly detected in healthy individuals at risk for IBD, but their significance is still unclear. It has been suggested that, as in celiac disease,³³ the presence of these antibodies may be a marker for a subclinical disease state.³² The potential high frequency of seropositivity in healthy individuals and its unclear significance are factors limiting population screening programs.

In conclusion, we found that serologic testing for ANCAs and ASCAs together identifies about 70% of children with IBD. High-titer ANCAs are spe-

cific for UC, whereas ASCAs are specific for CD. The unclear significance of positive test results in family members who are free of symptoms precludes screening of at-risk populations. Further study is necessary to determine the optimal clinical utility of ANCA and ASCA testing in children with known or suspected IBD.

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Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role

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Abstract

Background—Perinuclear antineutrophil cytoplasmic autoantibodies (pANCA) are a well recognised marker for ulcerative colitis. Antibodies to oligomannosidic epitopes of the yeast *Saccharomyces cerevisiae* (ASCA) are a new marker associated with Crohn's disease.

Aims—To assess the value of detecting pANCA and/or ASCA for the diagnosis of ulcerative colitis and Crohn's disease.

Methods—Serum samples were obtained from 100 patients with Crohn's disease, 101 patients with ulcerative colitis, 27 patients with other miscellaneous diarrhoeal illnesses, and 163 healthy controls. Determination of pANCA and ASCA was performed using the standardised indirect immunofluorescence technique and an ELISA, respectively.

Results—The combination of a positive pANCA test and a negative ASCA test yielded a sensitivity, specificity, and positive predictive value of 57%, 97%, and 92.5% respectively for ulcerative colitis. The combination of a positive ASCA test and a negative pANCA test yielded a sensitivity, specificity, and positive predictive value of 49%, 97%, and 96% respectively for Crohn's disease. Among patients with miscellaneous non-inflammatory bowel disorders, three were ASCA positive and two were pANCA positive. One control was ASCA positive. The presence of ASCA in patients with Crohn's disease was associated with small bowel involvement.

Conclusion—ASCA and pANCA are strongly associated with Crohn's disease and ulcerative colitis, respectively. Combination of both tests could help the diagnosis of inflammatory bowel disease.

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Keywords: Crohn's disease; ulcerative colitis; antineutrophil cytoplasmic autoantibodies; anti-Saccharomyces cerevisiae mannan antibodies

Inflammatory bowel diseases (IBD) are subdivided into ulcerative colitis (UC) and Crohn's disease (CD). Several lines of evidence suggest that CD and UC are different diseases. However, some patients (10-12%) cannot be easily classified into either and a final diagnosis

of indeterminate colitis is made.^{1,2} Making an earlier, more accurate diagnosis of IBD is important as the management of CD and UC is different, especially when surgery is planned. A search for serological tests to differentiate CD from UC has been underway for a long time. An ideal serological marker should have high sensitivity, high specificity, and high predictive values.

A subset of antineutrophil antibodies, commonly referred to as perinuclear antineutrophil cytoplasmic autoantibodies (pANCA) has been reported in sera from patients with IBD. The prevalence of pANCA varies from 40% to 80% in UC and from 0% to 20% in CD.³⁻⁶ Since 1988, systemic antibodies against the yeast *Saccharomyces cerevisiae* have been reported in sera from patients with CD. Although great variation was found both in patients' antibody responses and in the relative antigenicity of different strains, these antibodies have been associated with CD and not UC.⁷⁻¹¹ We recently showed that this serological response recognises mannose sequences in the cell wall mannan of *S cerevisiae* strain Su1 (formerly *S uvarum* 1, a species now classified within *S cerevisiae*).¹² Using the crude mannan from this strain as an antigen in an enzyme linked immunosorbent assay (ELISA), we found that testing for the presence of anti-*S cerevisiae* mannan antibodies (designated ASCA) was 64% sensitive and 77% specific for discriminating CD from UC and 89% specific for distinguishing CD from controls.¹²

In the present study, the association between pANCA and UC and ASCA and CD was evaluated by single or combined use of these tests. The relation between serological test results and clinical parameters of both diseases was also studied.

Patients and methods

PATIENTS

Serum samples were obtained from 100 patients with CD and 101 patients with UC, all unrelated. Diagnosis was based on the usual criteria.¹ Table 1 summarises their clinical data. Disease activity was assessed using the Crohn's disease activity index (CDAI).¹³ In patients with UC, disease was regarded as quiescent when quiescent or mildly active, or active when moderately active or severe according to the Truelove and Witts index.¹⁴ Patients who had previously been operated on were held to have active disease if there was clinical, biological, or

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Table 1 Clinical details of patients with Crohn's disease and ulcerative colitis

	Crohn's disease	Ulcerative colitis
No of patients	100	101
M/F	39/61	51/50
Mean age (y)	30.1	40.5
Disease duration (y)		
<5	60	40
5-10	29	23
>10	11	38
Active/quiescent	47/53	28/73
Disease location		
Small bowel	21	
Colon	37	
Small bowel and colon	42	
Distal		8
Left		21
Pancolic		19
Previous colectomy		53
Previous surgery	21	53
Medical treatment		
Sulphasalazine or mesalazine	26	20
Corticosteroids	9	11
Immunosuppressive drugs	13	1
Other treatment	9	0

endoscopic evidence of activity. We also studied sera from 27 patients with other miscellaneous colitides/diarrhoeal illnesses. These patients consisted of seven with collagenous colitis, six with acute self limited colitis, two with eosinophilic colitis, two with chronic radiation proctitis, two with subacute colonic schistosomiasis (*Schistosoma haematobium*), two with infectious colitis, one with pseudomembranous colitis, one with lymphocytic colitis, one with acute diverticulitis, one with systemic mastocytosis presenting with colonic mucosal involvement, one with coeliac disease, and one with sarcoidosis with colonic involvement. Sera from 163 healthy hospital staff members and blood donors without any history of gastrointestinal disease or familial history of IBD were used as controls.

All serum samples were stored at -40°C until assayed. Investigators had no knowledge of the diagnosis or clinical features at the time serological tests were conducted.

ANCA INDIRECT IMMUNOFLUORESCENCE ASSAY

Determination of pANCA was performed by an indirect immunofluorescence technique on ethanol fixed leucocytes according to the first International Workshop on ANCA.¹⁵ Fluorescein isothiocyanate conjugated rabbit antihuman IgG (specific for γ chains) (Dako, Glostrup, Denmark) was used. Patient sera were screened at a dilution of 1/20 in phosphate buffered saline. All slides were assessed by two well trained observers in a blinded fashion.

ASCA ELISA

Antigen consisted of phosphopeptidomannan (PPM) extracted from yeast cells from cultures in bioreactors. ELISA was performed as previously described.¹² Briefly, plates were coated with 100 μl of PPM at a concentration of 1 $\mu\text{g}/\text{ml}$ in sodium carbonate buffer (60 mM, pH 9.6), for one hour at 37°C and overnight at 4°C , in moist chambers, and then washed four times in TNT (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Patient sera

were diluted 1/1000 in TNT and tested in duplicate. Alkaline phosphatase labelled goat antihuman immunoglobulin (IgG, IgA, IgM; H and L chains; Zymed, Biosoft, Paris, France) was diluted 1/3000 in TNT. A colour reaction was obtained by using substrate Biotrol ELA 405 (Biotrol, Paris, France) for alkaline phosphatase. The plates were read at 405 nm on an Immunotech (Luminy, France) automatic reader. A coefficient of variation of less than 2% corresponded to repeatability of optical density values on a single microtitre plate. Each new set of experiments involved sera from four patients exhibiting graded ASCA levels as controls. Interseries reproducibility of ASCA values showed a coefficient of variation of less than 5%.

In our previous study,¹² we described the standardisation procedure which was designed to avoid variations in individual values observed between series of the immunological assay. Briefly, this involved the use of a standard consisting of a pool of CD patient sera strongly reacting with *S. cerevisiae* mannan. Each set of experiments involved six dilutions of the standard (1/500–1/32 000) from which a standard curve was derived. The highest absorbance (saturation) observed at 405 nm, was arbitrarily defined as 100% reactivity. Results of individual sera were expressed as a relative reactivity extrapolated from the standard curve and calculated by the ELIOT program (Immunotech, Luminy, France). The upper limit of normality, which gave the best compromise between sensitivity and specificity for CD versus UC, was determined following the establishment of receiver operating characteristics (ROC) curves. Curves were fitted using the SAS program, which indicated for each value the discriminating capacity in sensitivity and specificity.¹⁶ This was then used to determine the threshold of the test (3.12%).

STATISTICAL ANALYSIS

Sensitivity was defined as the probability of a positive test result in a patient with the disease under investigation. Specificity was defined as the probability of having a negative result in a patient without the disease under investigation. The positive predictive value was defined as the probability of being affected with the disease in a patient with a positive test result. It depends on the prevalence of the disease in the population under investigation and can be determined from a formula based on the Bayes theorem of conditional probability.¹⁷ The prevalences of CD and UC in Northern France are not known, but the incidences per 100 000 population are 4.9 and 3.2, respectively.¹ To calculate positive predictive values, we took the ratio of incidences of CD and UC to represent the ratio of their prevalences. When studying the relation between test results and clinical parameters, the χ^2 test or Fisher's exact test was used when appropriate, and a multivariate analysis was performed using a logistic regression model. Significance was assigned to any probability value less than 0.05.

Table 2 Test results for diagnosing either ulcerative colitis or Crohn's disease in patients with inflammatory bowel disease

Test	Ulcerative colitis (n=101)	Crohn's disease (n=100)	Sensitivity (%)	Specificity (%)	PPV (%)
pANCA+	66	15	65	85	74*
ASCA+	12	61	61	88	89†
pANCA+ASCA+	58	3	57	97	92.5*
pANCA-ASCA+	3	49	49	97	96†

*For ulcerative colitis; †for Crohn's disease.
PPV, positive predictive value.

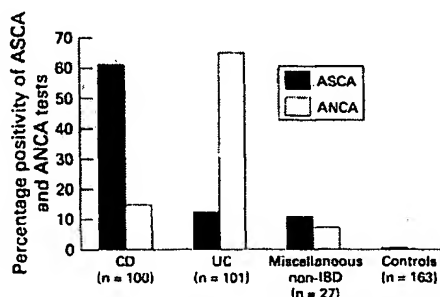


Figure 1 ASCA and ANCA results in the different study groups.

Results

Figure 1 shows perinuclear ANCA and ASCA results in the different groups. Table 2 shows performances of both tests used either singly or combined in 201 patients with IBD. Among patients with UC, no relation was observed between the presence of pANCA and any clinical parameter including site of disease, disease activity, treatment, or previous surgery (data not shown). Age at diagnosis was significantly less in patients with CD who were ASCA positive than in patients who were negative: 21 years (median), 10–59 (range) versus 24 years (15–47) ($p < 0.05$). Percentages of ASCA positivity were 62%, 46%, and 74% in patients with small bowel involvement, pure colonic disease, and both small bowel and colon involvement, respectively. The prevalence of ASCA was significantly higher in patients with small bowel involvement (with or without colonic disease) than in patients with pure colonic disease: 70% versus 46% ($p < 0.05$). The presence of ASCA was independent of disease activity and treatment. In the multivariate analysis, only small bowel location was significantly associated with ASCA positivity (adjusted odds ratio 2.7; 95% confidence interval 1.1–6.7; $p = 0.027$). Patients with CD who were pANCA positive had no particular clinical features compared with patients who were pANCA negative. Patients with UC who were ASCA positive had no particular clinical features compared with patients who were ASCA negative.

Among 27 patients with miscellaneous non-IBD disorders, one with eosinophilic colitis was ASCA positive and pANCA positive, two were ASCA positive and pANCA negative (one with radiation proctitis, one with sarcoidosis) and one was ASCA negative and pANCA positive (collagenous colitis). One of the 163 controls was ASCA positive.

Discussion

In the present study, we assessed the prevalence of ASCA and pANCA in a large population and their value for differentiating between UC and CD and between IBD and other colitides. At present, it appears that ASCA and pANCA are strongly associated with CD and UC, respectively.

Our percentages of serum samples from patients with UC and CD which were pANCA positive are comparable to the data reported in Western Europe.¹⁸ Overall sensitivity (61%) and specificity (88%) of ASCA for CD in this study were similar to those reported in a smaller series of adult patients,¹² and more recently in a paediatric series.¹⁹ After combining the two tests, sensitivity dropped by approximately 10%, as would be expected, but specificity increased to more than 95% yielding a very high positive predictive value for this combination. Among patients with miscellaneous non-IBD disorders, only three were ASCA positive and two were pANCA positive. These tests could thus be useful in clinical practice to differentiate between IBD and other colitides. The clinical relevance of serological tests also relates to differentiation of CD from UC in patients with colitis. Combination of both tests yielded high positive predictive values for the diagnosis of either UC or CD, which could make them usefully applicable to individual patients. In our study population of 37 patients with pure colonic CD, sensitivity dropped to 45% for ASCA and to 32% for the association pANCA negative/ASCA positive (12/37). However, the high positive predictive value would allow an accurate diagnosis in one third of these patients.

Increasing evidence supports the concept of clinical and genetic heterogeneity in IBD and serum immune markers have been used to characterise subgroups of patients.²⁰ The presence of pANCA in UC was independent of disease activity and extent; pANCA were also present in patients who had been operated on, confirming previous reports.^{1,8,21,22} Vasiliauskas *et al* reported that 100% of patients with CD who were pANCA positive had "UC like features".²¹ More recently the same group reported that there was a negative correlation between mean ANCA level and ASCA expression and that 100% of DNase sensitive pANCA/ASCA negative patients with CD had the "UC like" phenotype.²⁴ This suggests that a phenotypically distinct group of CD could be defined by their pANCA and ASCA status. In the present study, the presence of pANCA could not be associated with particular clinical features, thus confirming our previous report.²⁵ However, the techniques used to identify ANCA were different in Vasiliauskas *et al*'s study and ours. This further underscores the need for international standardisation for ANCA determination in IBD.^{25,26}

Patients with CD who were ASCA positive were younger than those who were ASCA negative, and the prevalence of ASCA was significantly associated with small bowel involvement. Using multivariate analysis, only small bowel involvement was significantly

associated with ASCA positivity. However, age and disease location are correlated: in this series, patients with small bowel involvement were younger at diagnosis than patients with pure colonic disease. The literature supports the concept of more small bowel disease in childhood and adolescent series and more colonic disease in older onset populations.^{27,28} Other series are needed to confirm whether ASCA represent a serological marker of a clinical subgroup with younger age at diagnosis and more frequent small bowel involvement.

The present study has shown that the combined use of ASCA and pANCA could differentiate CD from UC and other colitides although the relatively low prevalence of ASCA in patients with CD colitis may limit its clinical usefulness. Assaying for these markers may reveal clinical subgroups. However, since various prevalences of serological markers, such as pANCA, have been observed worldwide,³⁻⁸ it is recommended that multicentre, prospective studies be conducted.

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Diagnostic Accuracy of Serological Assays in Pediatric Inflammatory Bowel Disease

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See editorial on page 1006.

Background & Aims: Accurate serological assays are desirable for the diagnosis of inflammatory bowel disease (IBD) types in the pediatric age group. The aim of this study was to test the diagnostic accuracy of modified assays for perinuclear (p) antineutrophil cytoplasmic antibodies (ANCAs) and anti-*Saccharomyces cerevisiae* antibodies (ASCAs) in patients with pediatric ulcerative colitis (UC) and Crohn's disease (CD) and in those without IBD. **Methods:** With observers blinded to patients' diagnoses, serum specimens were analyzed for immunoglobulin (Ig) A and IgG ASCAs and ANCAs by enzyme-linked immunosorbent assay. The perinuclear location of ANCAs visualized by indirect immunofluorescence was confirmed by its disappearance after administration of deoxyribonuclease. **Results:** IgA and IgG ASCA titers were significantly greater and highly specific for CD (95% for either, 100% if both positive). pANCA was 92% specific for UC and absent in all non-IBD controls. The majority of patients with CD positive for pANCA had a UC-like presentation. Disease location, duration, activity, complications, and treatment with immunosuppressive drugs did not have an impact on the ASCA or pANCA assay results. After resection, UC patients remained pANCA positive, in contrast to patients with CD, in whom ASCA titers decreased toward normal values postoperatively. **Conclusions:** ASCA and pANCA assays are highly disease specific for CD and UC, respectively. These serological tests can assist clinicians in diagnosing and categorizing patients with IBD and may be useful in making therapeutic decisions.

As in adults, the clinical presentation of inflammatory bowel disease (IBD) in children depends largely on the site and extent of the mucosal inflammation. However, the diagnosis of IBD is more often delayed in children because of the frequency of nonspecific symptoms at the onset of disease.¹ Reliable serological screening tests would potentially be helpful in identifying

children and adolescents with IBD, resulting in earlier diagnoses.

Crohn's disease (CD) and ulcerative colitis (UC) are generally considered to be distinct forms of IBD. Yet, their symptoms and clinical presentations commonly overlap, and their discrimination in cases limited to the large bowel may be problematic.² Among patients with CD, a defined subgroup with a UC-like presentation has been described, illustrating the similarities of these diseases.³ To distinguish these entities from each other and especially from self-limited intestinal inflammation, various clinical, radiological, endoscopic, and histopathologic criteria have been put forth. Nevertheless, a certain proportion of cases (10%–15%) defy clear categorization even after colectomy and are commonly referred to as indeterminate colitis (IC).^{1,2,4} In the pediatric literature, IC is the term applied to cases of IBD limited to the colon with features suggestive of both UC and CD, without restricting the definition to patients who have undergone colectomy.⁴ Furthermore, a diagnosis in a patient initially identified as having UC may, over time, be "switched" to CD in view of the extension of disease.^{5,6} Accurate, noninvasive tests to distinguish such cases would be very helpful in such circumstances.

In UC, perinuclear (p) antineutrophil cytoplasmic antibodies (ANCAs) have been reported to be present in 60%–80% of cases, with a high degree of disease specificity.^{7,8} Main et al. first identified increased titers of anti-*Saccharomyces cerevisiae* antibodies (ASCAs) in the sera of adult patients with CD compared with those with UC or healthy controls.⁹ These antibodies are directed against distinct oligomannosidic epitopes of this yeast, as recently characterized by Sendid et al.¹⁰ Recent technical

Abbreviations used in this paper: ANCA, antineutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody; CD-DX-1, Crohn's disease diagnostic system; DNase, deoxyribonuclease; ELISA, enzyme-linked immunosorbent assay; EU, ELISA unit; IC, indeterminate colitis; p, perinuclear; UC-DX-1, ulcerative colitis diagnostic system.

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advances in these assays have allowed us to enhance the disease-specific accuracy of the ASCA and pANCA diagnostic systems.¹¹

This study was designed to test the diagnostic accuracy of these noninvasive tests, with special regard to their ability to discriminate pediatric patients with CD and colitis from those with UC and from non-IBD controls. We also examined the effect of disease location, duration, activity, complications, and medical as well as surgical treatments on ASCA and pANCA assay results.

Materials and Methods

Study Population

Serum was collected from pediatric patients attending the Ste-Justine Hospital gastroenterology inpatient service and IBD clinics. The selection of patients for this study was made on the basis of an absolute diagnosis in well-defined ("reagent-grade") patients ($n = 173$) who had undergone a complete diagnostic work-up for IBD. Furthermore, serum was collected from 36 consecutive patients who were prospectively assessed to rule out IBD. In all, 252 specimens from 209 consenting subjects were collected. All serum samples were coded so that analyses could be performed with the experimenter blinded to the patients' diagnoses. The study protocol was approved by the Ste-Justine Hospital Ethics Committee.

Clinical information for each patient was collected by chart review independently by two investigators unaware of the results of the antibody profiles. According to their concordant diagnosis established by standard clinical criteria and with endoscopic, histopathologic, and radiographic confirmation,⁴ the patients with IBD were classified as CD ($n = 130$) or UC ($n = 35$). A third group consisted of patients ($n = 11$) whose initial endoscopic and histopathologic diagnosis was that of an indeterminate colitis (IC), based on the criteria of Chong et al.¹² Among these, further investigations over a period of 0.5–4 years led to a diagnosis of CD and UC ($n = 1$ case each); these

patients were thus included in their respective CD and UC groups. The remaining 9 patients constituted our IC group. The control group ($n = 78$) without evidence of IBD consisted of patients with untreated celiac disease ($n = 25$), eosinophilic colitis ($n = 16$), irritable bowel syndrome ($n = 16$), acute self-limited bacterial colitis ($n = 10$), peptic ulcer disease ($n = 3$), autoimmune enteropathy ($n = 2$), gastroesophageal reflux ($n = 2$), or antral stenosis, intestinal pseudo-obstruction, polyposis, and an ischioanal fistula ($n = 1$ each). Further characterization of these patient groups is shown in Table 1. Disease activity for the group with CD was calculated by using a modified Harvey-Bradshaw index¹³ and by the Truelove and Witts scale for UC.¹⁴

CD Diagnostic System

The presence of ASCA was determined by a fixed enzyme-linked immunosorbent assay (ELISA; Prometheus Laboratory, San Diego, CA). Briefly, microtiter plates were coated with phosphopeptidomannans from the yeast *Saccharomyces uvarum*. Control and coded samples were added at a 1:100 dilution. Bound antibodies were labeled by alkaline phosphatase-conjugated goat anti-human immunoglobulin (Ig) G. After the addition of *p*-nitrophenol, specific absorbance was measured at 405 nm. The absorbance of each sample was evaluated and assigned ELISA unit (EU) values relative to the absorbance of a pool of sera collected from well-characterized patients with CD. The standard pool was arbitrarily assigned the value of 100 EU/mL. Results of the CD diagnostic system (CD-DX-1) panel were positive if either IgG or IgA or both were positive. Before this study, the cutoff for positivity was determined at Prometheus Laboratory on the basis of results in well-defined patients with CD and was set at 20 and 40 EU/mL for IgA and IgG ASCA, respectively.

UC Diagnostic System

The presence of ANCA was first screened for by means of a fixed neutrophil ELISA (Prometheus Laboratory) as described previously.¹⁵ Briefly, microtiter plates were coated

Table 1. Characterization of the Patient Groups Studied

	CD		UC		IC	Non-IBD
	CD-DX-1 ⁺	CD-DX-1 ⁻	UC-DX-1 ⁺	UC-DX-1 ⁻		
n	71	59	20	15	9	78
Mean age (yr)	14.3 (10–21)	13.8 (1–21)	13.6 (5–17)	12.1 (8–18)	13.8 (8–17)	6.5 (0.1–20)
Disease duration (mo)	22.4 (1–96)	22.0 (0–70)	15.7 (0–52)	19.8 (2–54)	15.3 (10–52)	—
Active disease	11	10	2	0	1	—
Small bowel involvement	19	15	—	—	0	—
Large bowel involvement	8	11	20	15	9	—
Small and large bowel involvement	44	33	—	—	0	—
Complications	16	6	3	4	2	0
Surgery	4	7	3	2	1	0
Corticosteroids	37	31	5	6	5	0
6-Mercaptopurine	8	4	0	0	0	0

NOTE. In CD, a Harvey-Bradshaw index of >5 was defined as active disease; disease activity in children with UC was determined by means of the Truelove index. As noted in Materials and Methods, disease location was based on radiological, histological, and endoscopic findings. Complications included fistulae, perforation, stenosis, or obstruction in children with CD, or arthritis, pericarditis, or autoimmune thyroiditis in children with UC.

+, positive; —, negative.

with 2.5×10^5 methanol-fixed neutrophils per well. After nonspecific antibody binding was blocked with 0.25% bovine serum albumin, control and coded sera were added at 1:100 dilutions. Neutrophil-bound antibody was labeled by alkaline phosphatase-conjugated goat anti-human IgG. After the addition of *p*-nitrophenol, specific absorbance was measured at 405 nm. Before this study, the cutoff for positivity was determined by positive controls from well-defined patients with UC (mean, 13.9 EU/mL) at Prometheus Laboratory.

Indirect immunofluorescence staining was then performed on ANCA ELISA-positive samples to determine whether a predominantly perinuclear (pANCA) or cytoplasmic (cANCA) staining pattern was present. Briefly, methanol-fixed neutrophils on glass slides were incubated with the coded samples (1:20 dilution). Specific binding was visualized by fluorescence microscopy after the addition of fluorescein-labeled anti-human IgG. The specificity of the perinuclear staining pattern in UC was finally confirmed by its disappearance after deoxyribonuclease (DNase) treatment of the neutrophils (Prometheus Laboratory). Results of the UC diagnostic system (UC-DX-1) panel were considered positive when both the ANCA titer was above the cutoff and the indirect immunofluorescence revealed a perinuclear binding of ANCA that disappeared after DNase treatment.

Statistical Analysis

All variables were tested for normal distribution by means of the David, Pearson, and Stephens test. When necessary, a log transformation was performed to obtain a normal distribution. Analysis of variance was used for comparison of IgA ASCA, IgG ASCA, and ANCA titers between groups. Adjustment for multiple comparisons was made using the Bonferroni correction. χ^2 tests were used to compare qualitative variables between groups. A *P* value of <0.05 was considered significant.

Results

CD-DX-1 Assay System

Comparison of ELISA results showed that the mean levels for both IgA and IgG ASCA (Figures 1 and 2)

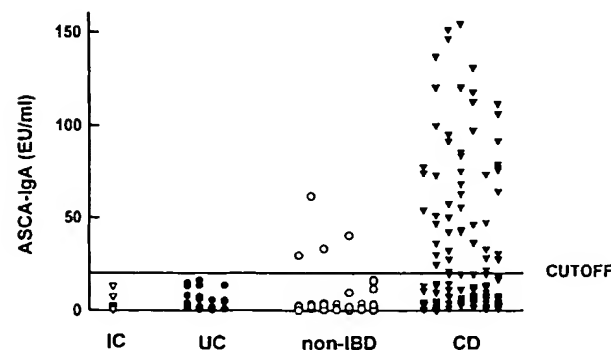


Figure 1. Scattergram showing IgA ASCA values as determined by ELISA in children with CD vs. children with UC, IC, and non-IBD controls.

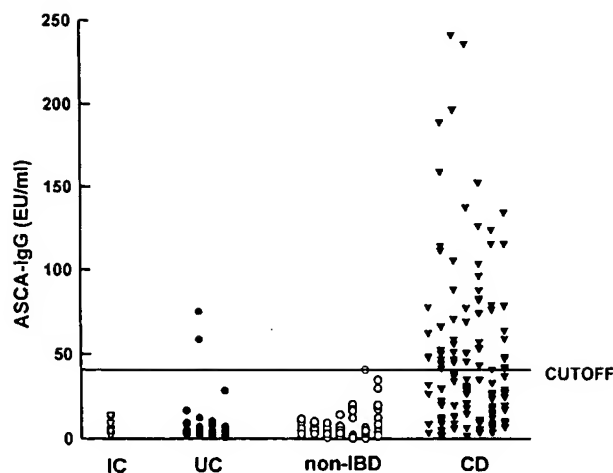


Figure 2. Scattergram showing IgG ASCA values as determined by ELISA in children with CD vs. children with UC, IC, and non-IBD controls.

were markedly higher in CD than the UC and non-IBD groups ($P < 0.001$). Moreover, as shown in Tables 2–4, the CD-DX-1 assay system (ASCA IgA and/or IgG) proved to be highly specific (95%) for CD, with 71 of 130 (55%) patients with CD being positive, compared with only 2 of 35 (6%) with UC, and 4 of 78 (5%) without IBD ($P < 0.005$). The combination of IgA and IgG ASCA positivity was 100% specific for CD. Only 1 of 11 patients initially classified as IC had a positive IgA ASCA titer. In the follow-up on this particular patient, a definitive diagnosis of CD was made 4 years after the initial blood sample was taken. Therefore, this patient was included in the CD group. None of the untreated celiac patients was ASCA positive.

UC-DX-1 Assay System

In distinct contrast, ELISA ANCA levels were significantly elevated in UC (Figure 3). Using indirect immunofluorescence with DNase, this assay system was highly specific (92%) for UC (Tables 2–4). Among patients with UC, 20 of 35 (57%) had a positive pattern compared with 17 of 130 (13%) and 0 of 78 (0%) children in the CD and non-IBD groups, respectively ($P < 0.005$). Antineutrophil cytoplasmic antibodies were present in some children in the CD and non-IBD groups. However, the perinuclear location characteristic of UC was not found in any non-IBD patients. Among the IC group, the 1 patient whose follow-up led to the diagnosis of UC was pANCA positive. Of the remaining 9 patients with IC, 6 had ANCA titers above the cutoff for positivity (Table 2). In 2 of these patients, the immunofluorescence after DNase treatment showed a true perinuclear staining pattern. Among the 17 patients with CD who were pANCA positive, 10 (59%) presented characteristic

Table 2. Results of ASCA and ANCA Assays in Pediatric Patients Evaluated for Possible IBD

Patient group	n	IgA ASCA+ n (%)	IgG ASCA+ n (%)	IgA or IgG ASCA+ n (%)	IgA and IgG ASCA+ n (%)	ANCA ELISA+ n (%)	pANCA indirect immunofluorescence after DNase n (%)
CD (all)	130	56 (43)	56 (43)	71 (55)	41 (32)	54 (42)	17 (13)
CD (colitis)	17	8 (47)	5 (29)	8 (47)	5 (29)	11 (65)	2 (12)
UC	35	0	2 (6)	2 (6)	0	31 (89)	20 (57)
IC	9	0	0	0	0	6 (67)	2 (22)
Non-IBD controls	78	4 (5)	0	4 (5)	0	8 (10)	0

+, positive.

UC-like symptoms, as recently defined by Vasiliauskas et al.³ This subgroup comprised 8 CD patients with pancolitis and 2 others with continuous, left-sided colitis. Interestingly, 7 of the 8 (88%) CD patients with pancolitis had very high ANCA titers, similar to that observed in the UC group (68.2 vs. 57.7 EU/mL, respectively). However, ANCA titers were significantly ($P < 0.001$) higher for the UC patients compared with the entire CD colitis subgroup. Furthermore, 5 of these 8 patients with UC-like pancolitis CD had a clear perinuclear ANCA immunofluorescent pattern.

Disease Location

As shown in Tables 1 and 2, no relationship was found between CD-DX-1 assay positivity (either IgA or IgG ASCA or both) and the site of CD inflammation (small vs. large bowel involvement, or both; $P = 0.7$). Comparing untreated celiac disease patients as small bowel inflammatory controls confirmed that the highly significant increase for both IgA ASCA ($P < 0.01$) and IgG ASCA ($P < 0.0001$) was restricted to the CD group. Patients with CD limited to the colon tended to have lower IgA and IgG ASCA compared with others with CD ($P = 0.3$; Figures 4 and 5), although their levels were significantly higher ($P < 0.005$) compared with all other patients with colitis (UC, IC, eosinophilic and acute,

self-limited colitis). The CD-DX-1 assay was highly specific for the CD colitis subgroup (Table 4): 96% with one ASCA positive and 100% with IgA and IgG ASCA positive (CD colitis vs. UC and non-IBD colitis). The UC-DX-1 assay was positive in 22% of patients with IC vs. 57% of patients with UC. Only 13% of the entire CD group (small and/or large bowel disease) and 12% of the CD colitis subgroup had a positive pANCA fluorescence pattern, confirmed by its disappearance after DNase treatment.

Disease Duration

No significant correlation was observed between the duration of symptoms in CD and the results of the CD-DX-1 assay (Table 1), excluding the 11 patients who had undergone surgery (22.9 months [range, 1–96 months] vs. 17.0 months [range, 0–70 months] for CD-DX-1-positive vs. -negative patients with CD; $P = 0.25$). Among children with a recent diagnosis of CD, 10 of 14 (71%) were positive for CD-DX-1. The results of this subgroup, tested during initial diagnostic workup, were not statistically different from those of the entire CD group ($0.5 < P < 0.7$). Among the 20 patients with CD who underwent repeated assays, 3 converted from ASCA negative to positive after a variable period (1 month to 5 years). Three others remained

Table 3. Diagnostic Accuracy of the Serological Tests to Distinguish Between CD, UC, and Non-IBD

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
IBD vs. non-IBD: CD-DX-1 or UC-DX-1 assay	55	95	96	50
CD vs. UC + non-IBD: CD-DX-1 assay (IgA or IgG)	55	95	92	64
CD vs. UC + non-IBD: CD-DX-1 assay (IgA and IgG)	32	100	100	56
UC vs. CD + non-IBD: ANCA ELISA	89	70	33	97
UC vs. CD + non-IBD: UC-DX-1 assay	57	92	54	93

Table 4. Diagnostic Accuracy of the Serological Tests to Distinguish Between CD Colitis, UC, and Non-IBD Colitides

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
CD colitis vs. UC + non-IBD colitis: CD-DX-1 IgA or IgG ASCA	47	96	73	87
CD colitis vs. UC + non-IBD colitis: CD-DX-1 IgA and IgG ASCA	29	100	100	87
UC versus CD colitis + non-IBD colitis: ANCA ELISA	89	78	70	92
UC vs. CD colitis + non-IBD colitis: UC-DX-1 assay	57	97	91	79

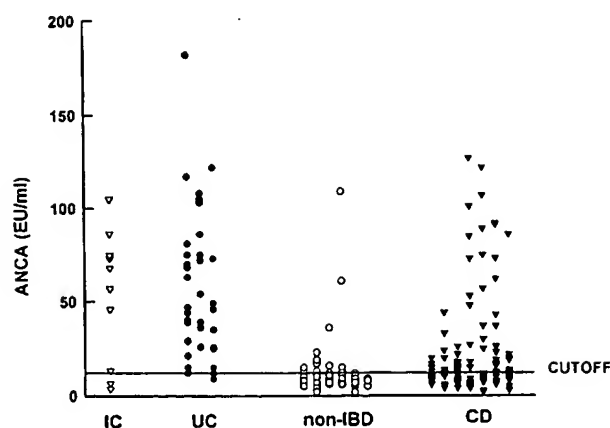


Figure 3. Scattergram showing ANCA titers as determined by ELISA in pediatric patients with UC, IC, and CD, and non-IBD controls.

CD-DX-1 negative; the others showed little variation (<25%) in ASCA titers over time. None of the initially ASCA-positive patients with CD converted to negative.

Similarly, disease duration did not influence the results of the UC-DX-1 assay system in patients with ulcerative colitis (mean, 15.7 and 19.8 months for UC-DX-1-positive and -negative groups, respectively; $P > 0.25$).

Disease Activity

We did not observe a statistically significant effect of disease activity on ASCA titers in the CD group or on ANCA results in the UC group. Among the patients with CD with active disease ($n = 21/119$, 18%), defined by a Harvey-Bradshaw index of >5 , mean ASCA IgG was 82 EU/mL vs. 66 EU/mL, and mean ASCA IgA was 28 EU/mL vs. 34 EU/mL for the subgroup in remission ($P = \text{NS}$). ASCA double positivity in CD did not correlate with higher disease activity.

Complications

Serum IgA and IgG ASCA titers were not different among CD patients with complications in the form of

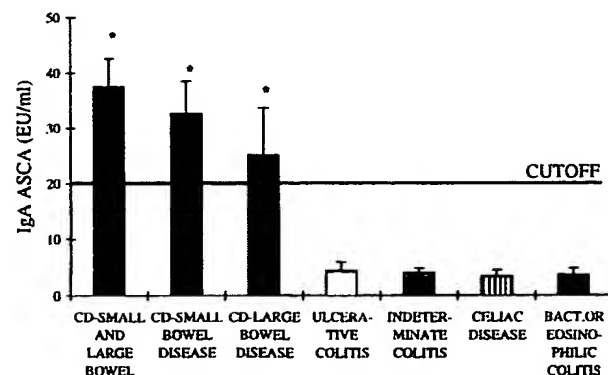


Figure 4. Effect of the site of inflammation on IgA ASCA titers (mean of each group \pm SD). * $P < 0.01$, CD vs. UC or non-IBD.

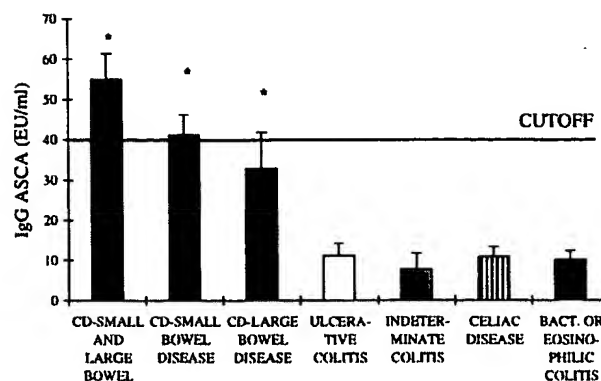


Figure 5. Effect of the site of inflammation on IgG ASCA titers (mean of each group \pm SD). * $P < 0.01$, CD vs. UC or non-IBD.

fistulae, perforation, stenosis, or obstruction ($n = 22$, 18%) compared with those without such complications (Table 1). Among the patients with UC, 7 presented with extraintestinal complications, including arthralgias ($n = 5$), pericarditis ($n = 1$), and autoimmune thyroiditis ($n = 1$). These associated autoimmune phenomena did not have an impact on the results of the UC-DX-1 assay.

Medical Therapy

The use of systemic corticosteroids (intravenous or oral) in CD ($n = 68$, 52%) did not influence the results of the CD-DX-1 assay; their use in UC ($n = 11$, 31%) also had no effect on the results of the UC-DX-1 assay (Table 1). Similarly, mesalamine or 6-mercaptopurine ($n = 12$) use did not have an impact on these results compared with untreated findings in patients with IBD.

Surgery

Among patients with CD, 11 of 130 (8%) had undergone a resection. In 7 of these, the postoperative CD-DX-1 assay results were negative. The mean interval between surgery and testing was 3 years in the CD-DX-1-negative group, whereas the 4 patients positive for ASCA were all analyzed immediately postoperatively (<4 days). Unfortunately, these patients were not part of a prospectively conducted study, and therefore no serum from before surgery was available for comparison. In the group with UC, 5 of 35 (14%) had undergone surgery. Except for 1 child with negative results of an ANCA panel despite high ANCA ELISA titers, the 4 others remained pANCA positive after colectomy (mean interval between surgery and the assay, 4 months; range, 1–18 months).

Discussion

A dramatic increase in the incidence of CD during childhood has been recognized over the past few decades,

with a trend toward an increasing number under the age of 10 years at the time of diagnosis.¹⁶⁻²⁰ Prompt recognition of IBD, whose incidence peaks in the second decade of life, has thus become increasingly important for physicians caring for children and adolescents. In this study, we show for the first time that the CD-DX-1 and UC-DX-1 assay systems are highly specific diagnostic tools in pediatric CD and UC, respectively. As shown in Table 3, the specificity and positive predictive values of the two assays in discriminating IBD vs. non-IBD patients were 95% and 96%, respectively. Furthermore, only children with CD were double-positive for IgA and IgG ASCA (100% specific). The combination of ANCA testing by ELISA followed by immunofluorescence and the additional confirmatory step using DNase treatment of neutrophils with bound antibodies, resulting in the disappearance of the perinuclear staining, increased the disease specificity of the assay in UC patients from 70% to 92%, in accordance with recent reports in adults.^{7,8,21} The combined use of both assay systems as an IBD panel gave an overall agreement of 68% with the final diagnosis (CD, UC, or non-IBD) on the basis of clinical, endoscopic, radiographic, and histopathologic criteria.

As in adults, the clinical presentation of IBD in the pediatric age group depends largely on the site and extent of the mucosal inflammation.¹ Insidious onset of abdominal pain, chronic diarrhea, and weight loss are hallmark symptoms of CD, whereas rectocolonic inflammation due to UC or CD usually presents more acutely with hematochezia. However, the diagnosis of IBD is often delayed in pediatric patients because of the nonspecific nature of symptoms.^{1,6} These include decelerated growth velocity, anorexia with fatigue, or pubertal delay. Furthermore, extraintestinal manifestations such as arthritis or fever are more frequent in children than in adults, potentially misdirecting the initial clinical evaluation.^{1,6} The results of this study suggest that this noninvasive IBD panel could be useful in suspected cases of CD and UC. In the subset of 36 patients prospectively evaluated for the possibility of IBD, 71% of patients with CD and 75% of those with UC had positive findings of a disease-specific panel at the time of their initial diagnostic workup. Although the CD-DX-1 and UC-DX-1 assays are imperfect, their sensitivities of the CD-DX-1 and UC-DX-1 assays are vastly superior to that reported for other commonly used biological disease markers, such as the rheumatoid factor (15%–20% sensitivity) or antinuclear antibody test (24%–66%) for juvenile rheumatoid arthritis.²² About two thirds of pediatric patients with CD have an elevated erythrocyte sedimentation rate.²³ However, this widely used biological marker lacks disease

specificity and is not useful in discriminating between CD and UC.

A major diagnostic dilemma in the pediatric age group results from the overlap between UC and CD limited to the large bowel. Most often, such cases are grouped as IC.^{1,4} In the present study, we classified our patients into well-defined CD or UC groups. The IC classification was used when the endoscopic findings and histopathologic features of the biopsy specimens did not clearly distinguish between the other two entities. We observed that 13% of children with CD were positive for pANCA, consistent with the 10%–30% rate in patients with CD who express serum pANCA in adult IBD cohorts.^{24,25} Looking at the CD-colitis subgroup, the percentage of pANCA-positive children (12%) was significantly inferior to that noted for the UC group (57%). Moreover, the UC-DX-1 assay proved to be highly specific (97%) for UC vs. CD and non-IBD colitis, with positive and negative predictive values of 91% and 79%, respectively (Table 4).

In our series, the majority (59%) of CD patients with pANCA immunofluorescence presented characteristic UC-like symptoms. Vasiliauskas et al.³ recently noted that all pANCA-positive adult patients with CD had UC-like symptoms. The observation of pANCA in young children with CD at the onset of their disease supports the concept of a biological marker in a distinct subgroup of patients with CD. Furthermore, positive pANCA titers in the majority of pediatric patients with UC who had undergone surgery persisted, in agreement with findings of other recent studies.²⁶ The absence of pANCA in acute, self-limited, or eosinophilic colitis indicates that this disease marker does not simply reflect the presence of mucosal inflammation but more likely the type of mucosal inflammation. Similarly, ASCA titers were negative in all of our untreated celiac patients, in contrast to results of a previous report using a different method in adults.²⁷

Among the group of children with IC, 22% had a positive UC-DX-1 panel vs. only 12% and 0% for the CD and non-IBD groups, respectively. These data suggest that children presenting with mild forms of UC are more likely to be classified initially as IC on clinical grounds. During follow-up, one of our IC pANCA-positive patients later proved to have UC.

The CD-DX-1 assay was able to clearly discriminate between CD colitis and IC, having 100% specificity for IgA and/or IgG ASCA positivity in CD patients with colitis. The only child with positive ASCA titers, initially classified as IC, was later confirmed to have CD. These data allow us to speculate that results of this assay, if positive, help in identifying and classifying patients with

CD who initially present with nonspecific colonic inflammation. Furthermore, the CD-DX-1 was 100% predictive value positive for CD colitis vs. UC and non-IBD colitis when the IgA and IgG ASCA were both positive.

In contrast to persistent pANCA results in patients with UC after colectomy, ASCA titers decreased toward normal values found in non-IBD and healthy controls when tested a few years after resection in pediatric patients with CD. This suggests the possibility that ASCA represents a humoral immune response to a luminal antigen, taken up across the inflamed, disrupted mucosal barrier. However, a prospective analysis of serum ASCA titers before and after surgery in a larger cohort of patients with CD is needed to confirm these findings. On the other hand, it is notable that patients with CD do not have higher antibody titers to *Candida* and other yeast organisms present within the gut lumen compared with healthy controls.²⁸ In view of negative ASCA titers in our untreated celiac patients, altered barrier function alone is an insufficient explanation. We did not observe a relationship between disease activity and ASCA positivity (Table 1). However, prospective testing serially over a defined period would have to be performed for firm conclusions on this issue to be drawn.

Our data on the combined use of the noninvasive CD-DX-1 and UC-DX-1 assays (Tables 2–4) suggest that they are helpful in the diagnosis of IBD in children. When the results of both tests are taken together, we observed a specificity of 95% and a positive predictive value of 96% for IBD. The diagnostic value of the combined use of these serological tests has been shown in a recent preliminary report in an adult cohort with IBD.²⁹ The high disease specificity of both diagnostic panels may be of particular assistance in making major therapeutic decisions, such as in patients with severe colitis. Positive CD-DX-1 and negative UC-DX-1 panel findings would favor the use of medical rather than surgical therapy in the nontoxic patient. Further prospective studies are needed to ascertain the predictive value and cost-effectiveness of using ASCA in combination with other laboratory markers in screening for IBD in patients with nonspecific symptoms and normal results of a physical examination.

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Anti-*Saccharomyces cerevisiae* Mannan Antibodies and Antineutrophil Cytoplasmic Autoantibodies in Greek Patients With Inflammatory Bowel Disease

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OBJECTIVES: The combined measurement of perinuclear antineutrophil cytoplasmic autoantibodies (pANCA) and anti-*Saccharomyces cerevisiae* mannan antibodies (ASCA) has recently been suggested as a valuable diagnostic approach in inflammatory bowel disease (IBD). The aim of this study was to assess the value of detecting pANCA and ASCA in the differentiation between ulcerative colitis (UC) and Crohn's disease (CD) in a Greek population with IBD.

METHODS: Sera were collected from 157 patients with IBD (97 with UC, 56 with CD, and four with indeterminate colitis) and 150 healthy controls. Determination of pANCA was performed by a standard indirect immunofluorescence technique on ethanol-fixed granulocytes and ASCA by an ELISA assay.

RESULTS: In patients with UC, sensitivity, specificity, positive predictive value, and negative predictive value of the pANCA test was 67%, 84%, 93%, and 46% respectively. These values did not change significantly when the combination of positive pANCA and negative ASCA was used. ASCA test in diagnosing CD yielded a sensitivity, specificity, positive predictive value, and negative predictive value of 39%, 89%, 54%, and 81%. The combination of pANCA negative and ASCA positive increased the positive predictive value to 77% and it was associated with small bowel disease.

CONCLUSIONS: A positive pANCA test in Greek patients has a diagnostic value in confirming a diagnosis of UC. Measurement of pANCA and ASCA together has a rather limited value in the differential diagnosis between UC and CD but may be of help in studying disease heterogeneity. (Am J Gastroenterol 2001;96:449–454. © 2001 by Am. Coll. of Gastroenterology)

INTRODUCTION

A search of serological markers to differentiate between ulcerative colitis (UC) and Crohn's (CD) disease has been

underway for >40 yr (1, 2). Several serological tests have been examined in patients with inflammatory bowel disease (IBD) including antipancreatic antibodies (3, 4), antierythrocyte antibody (5), antiendothelial cell antibody (6, 7), antibacterial/permeability-increasing protein antibodies (8), anti-p40 (40 kD) antibodies (9) perinuclear antineutrophil cytoplasmic autoantibodies (pANCA) (10–13), and anti-*Saccharomyces cerevisiae* mannan antibodies (ASCA) (14–17). Among all of these antibodies, pANCA and ASCA have been most extensively studied and have been suggested as possible diagnostic markers in IBD.

The pANCA represent a distinct subset of antineutrophil cytoplasmic autoantibodies with perinuclear staining by indirect immunofluorescence; they have been found in about 60–70% of UC patients and 5–10% in patients with CD (12, 13, 18). On the other hand, ASCA have been identified in raised titers in 60–70% of patients with CD, in 10–15% of UC patients, and in 0–5% of healthy controls (17, 19–21).

The measurement of pANCA or ASCA alone has been found to have limited clinical diagnostic value in IBD because of inadequate sensitivity to diagnose UC and CD. Recently, the combined measurement of pANCA and ASCA has been advocated as a valuable diagnostic approach in IBD (20–22).

The aim of the present study, therefore, was the evaluation of the diagnostic value of pANCA and ASCA, either alone or in combination in a well defined racially homogeneous Greek IBD population. The relationship of these antibodies with clinical parameters of UC and CD were also assessed.

MATERIALS AND METHODS

Patients

A total of 157 IBD patients followed-up at the Department of Gastroenterology of the University Hospital of Heraklion Crete were included in the study (Table 1). Of the UC patients 59 were men and 38 were women, with a mean age

Table 1. Clinical Details of the Patients Included in the Study

	UC	CD	Total
Number	97	56	153
Male	59	33	92
Female	38	23	61
Active	32	18	50
Inactive	65	38	103
Disease location			
UC			
Proctitis	22		
Left sided	41		
Extensive	34		
CD			
Small bowel		17	
Colon		20	
Small bowel and colon		19	
Disease type (CD)			
Stenotic		18	
Fistulizing		10	
Inflammatory		28	
Treatment*			
Salazopyrine	6	3	9
5-Aminosalicylic acid	76	35	111
Oral steroids	21	10	31
Topical steroids	12	2	14
Azathioprine	6	9	15
Methotrexate	0	3	3
Metronidazole	0	13	13
None	7	3	10

* Some patients received more than one drug.

of 49 yr and a mean disease duration of 7.8 yr. The CD patients included 33 men and 23 women with a mean age of 40 yr and a mean disease duration of 8.3 yr. Four cases were diagnosed as indeterminate colitis. IBD patients were compared with 150 healthy controls who were matched to the patient population for age and sex. These control subjects were recruited from among healthy blood donors, healthy visitors to hospital wards (Gynecology/Obstetrics and Orthopedics), and normal hospital personnel. Sera were collected from the 157 IBD patients and the 150 healthy controls. Diagnosis of UC and CD was based on standard criteria (23). Disease activity in CD was evaluated by using the Crohn's Disease Activity Index (CDAI) score (24) and in UC by the Truelove and Witts's index (25). All serum samples were stored at -70°C until assayed.

ANCA Indirect Immunofluorescence Assay

Determination of pANCA was performed by the standard indirect immunofluorescence technique on ethanol-fixed granulocytes (INOVA Diagnostics, San Diego, CA) and according to the First International Workshop on ANCA (26). All slides were always read by two independent observers who were not aware of the clinical diagnosis.

ELISA for ASCA

An ELISA for ASCA was performed as previously described from Sendid *et al.* (17). Briefly, phosphopeptidomannan was extracted from cultures of yeast cells and used as the antigen (19). Plates were coated with 100 μl of antigen

at a concentration of 1 $\mu\text{g}/\text{ml}$, diluted in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin, and incubated for 1 h at room temperature and overnight at 4°C . After three washes with PBS, 50 μl of patients sera diluted 1/50 in PBS was added to the wells in duplicate. After incubation for 1 h at room temperature, the plates were washed three times with PBS, and then 50 μl of alkaline phosphatase goat antihuman immunoglobulin (IgG, IgA, IgM; Sigma, St. Louis, MO) diluted 1/1000 in PBS was added to each well. The plates were incubated at room temperature for 1 h. After rewashing three times with PBS, 50 $\mu\text{l}/\text{well}$ of p-nitrophenyl phosphate (Sigma), 1 mg/ml in diethanolamine buffer, pH: 9.8, was added for 20–30 min in the dark at room temperature. The reaction was stopped by adding 50 $\mu\text{l}/\text{well}$ of 3N NaOH, and the absorbance was read at 405 nm in a microplate reader (Dynatech, Ashford, Middlesex, UK). The mean absorbance value (0.235) plus 3 standard deviations ($\text{SD} = 0.015$) of this asymptomatic population were calculated and used as the value to discriminate between positive and negative samples. This stringent cut off point was adopted to preclude the possibility of false positive results. Absorbance unit values greater than this mean plus 3 SD (0.28) were considered positive for ASCA antibodies, whereas absorbance unit values <0.28 were considered negative.

A second evaluation of our ASCA results in CD patients was performed in the Laboratory of Microbiology, University of Thessaly (investigator, A.N.M.), using the Quanta Lite IgG and IgA ASCA ELISA kits (INOVA Diagnostics). Samples were interpreted as positive (IgG or IgA antibody to *Saccharomyces cerevisiae* detected) if ASCA was ≥ 25 units.

Statistical Analysis

Sensitivity was defined as the probability of a diagnostic test being positive for a patient with the disease under investigation. Specificity was defined as the probability of a test being negative for a patient without the disease under investigation. These two probabilities are known as the operating characteristics of a diagnostic test.

The PPV of a test is the posterior probability of a patient affected by the disease under investigation, given that the test is positive. Similarly, the NPV is the posterior probability of a patient not affected by the disease under investigation, given that the test is negative. These two probabilities depend on the prevalence of the disease in the general population (prior probability) and are determined by the well known Bayes theorem.

When discriminating between two diseases such as UC and CD, both PPV and NPV depend on the ratio of the two prevalences. The prevalences of UC and CD in the area of Heraklion are not known, but the incidences per 100,000 population are 8.9 and 3.0, respectively (27, 28). The PPV and NPV were calculated using the ratio of incidences as an estimate of the ratio of their prevalences.

The relationship between serological markers and clinical

Table 2. Diagnostic Accuracy of the Serological Tests to Distinguish Between Ulcerative Colitis and Crohn's Disease

Test	Ulcerative Colitis (n = 97)	Crohn's Disease (n = 56)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
pANCA+	65	9	67	84	93*	46*
ASCA+	11	22	39	89	54†	81†
pANCA+ ASCA-	56	7	58	88	93*	41*
pANCA-ASCA+	3	17	30	97	77†	81†

* For ulcerative colitis.

† For Crohn's disease.

NPV = negative predictive value; PPV = positive predictive value; other abbreviations as in text.

parameters were studied using the χ^2 -test or Fisher's exact test where appropriate. A *p* value of < 0.05 was considered to be significant.

RESULTS

The pANCA were detected by indirect immunofluorescence on ethanol-fixed granulocytes in 65 of 97 (67%) samples from UC patients and in nine of 56 (16%) samples from CD patients (Table 2). Moreover, an atypical cytoplasmic staining pattern, which was different from the c-ANCA pattern characteristic of antiproteinase 3 antibodies, was found in four UC and two CD patients. Three of four samples from patients with indeterminate colitis (75%) were pANCA-positive. Only one of 150 (0.7%) samples from healthy controls was found positive for pANCA by indirect immunofluorescence. A total of 32 UC patients had active disease, whereas 65 patients had inactive disease; 69% of the first group and 66% of the second group were pANCA-positive (Table 3). The difference between the two groups was not statistically significant. In addition, no significant differences were found among the three groups classified according to disease localization. A total of 18 CD patients had active disease, whereas 38 patients were in remission. Positivity for pANCA was 17% in the first group and 16% in the second group (Table 3); pANCA-positivity was found in six patients with CD (30%) of the colon, in two patients with

CD of both small bowel and colon (11%) and in one patient with CD of the small bowel (6%). The prevalence of pANCA was significantly higher in patients with colonic involvement than in the other groups (*p* = 0.04).

Raised titers of ASCA were found in 11 of 97 UC patients (11%), in 22 of 56 CD patients (39%), and in two of 150 healthy controls (1.3%). None from the patients with indeterminate colitis were ASCA-positive. Among UC patients no relation was observed between ASCA positivity and the various clinical parameters (Table 4). Also in CD, the presence of ASCA was not related to disease activity and treatment. Percentage of ASCA positivity was 59%, 25%, and 37% in CD patients with small bowel involvement, colonic disease, and both small bowel and colon involvement, respectively (Table 4). Although there was a trend toward a higher ASCA positivity in patients with small bowel involvement, the difference among the localization groups was not statistically significant (*p* = 0.13). Re-examination of the sera of CD patients using Quanta Lite IgG and IgA ASCA ELISA kits (INOVA Diagnostics, San Diego, CA) showed ASCA positivity in 20 of the 56 CD patients (35.7%). Six cases were both IgG- and IgA-positive, six cases were IgG-positive only, and eight cases were IgA-positive. All negative cases with ELISA made in our laboratory were also negative with the commercially available kit. On the other hand, two ASCA-positive cases with our own ELISA were equivocal with the commercial kit.

Table 3. Relationship Between pANCA and Disease Activity and Localization

Disease	n	pANCA(+)	pANCA(-)
UC			
Total	97	65 (67%)	32 (33%)
Active	32	22 (69%)	10 (31%)
Nonactive	65	43 (66%)	22 (34%)
Proctitis	22	14 (64%)	8 (36%)
Left-sided colitis	41	27 (66%)	14 (34%)
Extensive colitis	34	24 (71%)	10 (29%)
CD			
Total	56	9 (16%)	47 (84%)
Active	18	3 (17%)	15 (83%)
Nonactive	38	6 (16%)	32 (84%)
Small bowel	17	1 (6%)	16 (94%)
Colon	20	6 (30%)	14 (70%)
Small bowel and colon	19	2 (11%)	17 (89%)

CD = Crohn's disease; UC = ulcerative colitis; other abbreviations as in text.

Table 4. Relationship Between ASCA and Disease Activity and Localization

Disease	n	ASCA (+)	ASCA (-)
UC			
Total	97	11 (11%)	86 (89%)
Active	32	4 (13%)	28 (87%)
Nonactive	65	7 (11%)	58 (89%)
Proctitis	22	2 (9%)	20 (91%)
Left-sided colitis	41	5 (12%)	36 (88%)
Extensive colitis	34	4 (12%)	30 (88%)
CD			
Total	56	22 (39%)	34 (61%)
Active	18	8 (44%)	10 (56%)
Nonactive	38	14 (37%)	24 (63%)
Small bowel	17	10 (59%)	7 (41%)
Colon	20	5 (25%)	15 (75%)
Small bowel and colon	19	7 (37%)	12 (63%)

Abbreviations as in Table 3.

A total of 52 IBD patients (25 UC and 27 CD) were found to be negative for both pANCA and ASCA, whereas five UC and two CD patients were found to be positive for both.

The values of sensitivity, specificity, PPV, and NPV of pANCA and ASCA, either alone or in combination, are shown for our IBD patients in Table 2. In patients with UC, the sensitivity, specificity, PPV, and NPV of the pANCA test were 67%, 84%, 93%, and 46% respectively. These values did not significantly change when the combination of pANCA-positive and ASCA-negative was used. Moreover, this phenotype was not different among the clinical subgroups of UC. The use of the ASCA test in diagnosing CD yielded a sensitivity, specificity, PPV, and NPV of 39%, 89%, 54%, and 81% respectively. The combination of pANCA-negative and ASCA-positive improved the PPV to 77%. This phenotype was not different between active and inactive CD, but it was associated with a small bowel disease localization (10 of 17 patients). No patient with only colonic CD was pANCA-negative and ASCA-positive.

DISCUSSION

In the present study we assessed the value of pANCA and ASCA, alone or in combination, for the diagnosis of IBD and for the differentiation between ulcerative colitis and Crohn's disease in a well defined Greek population. It seems that the pANCA test may have significant diagnostic value for UC with results (prevalence 67%) comparable to those of other European centers (12, 13). The prevalence of pANCA in our UC patients was found to be significantly higher in comparison to a previous study from another Greek area where the prevalence of pANCA was only 30% (29). The reason for this discrepancy is not clear. In our UC patients, pANCA were not related to the extent or activity of the disease—a finding similar to most of the previous reports (12, 13). In accordance with previous studies (30) the prevalence of pANCA in our CD patients was also significantly higher in patients with colonic involvement than in those with small bowel disease.

The prevalence of ASCA in CD patients was only 39% in our population, which was significantly lower than the reported prevalence of 60–70% in other studies (17, 19–21). There are two possible explanations for this discrepancy. Either our ELISA technique is not sufficiently sensitive (which seems rather unlikely, after confirmation of the results with the commercial ELISA) or, more probably, the phenotype of our patients is different from the phenotypes of patients from Northern Europe. The present study is the first on the prevalence of ASCA in Greek IBD patients. The prevalence of ASCA antibodies was weakly associated with the presence of small bowel involvement in CD patients, but this relationship did not reach statistical significance. No other relationship between ASCA and the clinical parameters of both diseases was found. It seems that ASCA has little value in the routine clinical assessment of CD patients,

but should be reserved for studies in which a more accurate definition of subgroups is needed.

In the small but clinically important population of patients with indeterminate colitis, the combined measurement of pANCA and ASCA might be more useful. However, in the present study we were unable to draw any conclusions because of the small number of patients with indeterminate colitis.

Combination of both tests did not increase the PPV for UC compared to pANCA alone whereas the use of the combination pANCA negative and ASCA positive raised the PPV for CD from 54% (ASCA alone) to 77%. The diagnostic role of the combination of pANCA and ASCA was assessed in one study in adults (20) and in two pediatric series (21, 22). Although in two of the studies pANCA and ASCA were found to be highly disease-specific for UC and CD, in the study of Hoffenberg *et al.* (22) testing for both antibodies did not achieve the same degree of discriminating power, which is in accordance with our results. The differences between results of serological testing in IBD, including our data, can perhaps be explained by the lack of the standardization of the methodology or by disease heterogeneity. There are also differences in the controls used in these studies. In some of the studies, subjects other than IBD and irritable bowel syndrome patients have been used as controls. However, in these studies, no significant difference was found in either specificity or sensitivity of the tests for patients compared with true healthy controls. Therefore, in our study we used matched healthy controls because we believed that they could not considerably bias our results in achieving a higher measure of diagnostic specificity of the ASCA test.

There is ample evidence to support the concept of clinical and genetic heterogeneity in IBD (31), and various serological markers have been used to characterize subgroups of patients. Both pANCA and ASCA have been proposed as subclinical genetic markers and a familial expression of them has been observed (32, 33). However, positivity and antibody titers of pANCA were not related to the extent or activity of the disease, and they persist even after colectomy (34), being often predictive of pouchitis (35). The presence of ASCA also seems to be independent of disease activity. There is evidence that pANCA and ASCA could be useful in identifying distinct subgroups within UC and CD. Positive pANCA in CD patients has been postulated as a marker for a UC-like phenotype (30), and our data support this hypothesis. Positive pANCA in UC patients have been suggested as a risk factor for pouchitis after ileal pouch anal anastomosis (35). The expression of pANCA has also been proposed as a prognostic factor of more aggressive left-sided UC (36) that tends to be resistant to immunomodulatory therapy and often requires surgical intervention. In our study, no association between a positive pANCA and either active or more aggressive disease was found; however, the number of patients with treatment resistant left-sided disease was too small (five cases, three pANCA-positive) to

draw any final conclusion. In CD patients, ASCA positivity has been linked to small bowel disease (20) and our data are also in agreement with this observation. It has also been suggested that treatment decisions should take into account the pANCA and ASCA positivity; however, many more clinical studies are required before such a recommendation is accepted.

The present study has shown that a positive pANCA test in Greek patients has a diagnostic value in confirming a diagnosis of UC. In contrast ASCA positivity has a rather limited value in diagnosing CD. Overall, about one third of the patients in this study could not be accurately categorized based on serological testing. The combination of pANCA and ASCA assays did not offer the high specificity observed in other studies. It seems that the combination of both tests in our population has limited clinical value. ASCA phenotype has been suggested to show ethnic variation, and our findings point to this direction. Although the serological tests have been suggested as possible tools for screening the general population and groups at high risk for IBD, our results do not support this.

In conclusion, we found that serological testing for pANCA and ASCA in Greek IBD patients did not achieve the sensitivity necessary for population screening. However, these tests may be helpful in identifying distinct disease subgroups and may contribute to treatment in the future.

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